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CYTOPLASMIC INHIBITION OF GENE EXPRESSION
AND EXPRESSION OF A FOREIGN PROTEIN
IN A MONOCOT PLANT BY A PLANT
VIRAL VECTOR

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CYTOPLASMIC INHIBITION OF GENE EXPRESSION AND EXPRESSION OF A FOREIGN PROTEIN IN A MONOCOT PLANT BY A PLANT VIRAL VECTOR

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FIELD OF THE INVENTION

The present invention relates to the field of molecular biology and genetics. Specifically, the invention relates to the use of recombinant viruses and recombinant viral vectors in expressing foreign peptides and/or regulating gene expression in a plant host.

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BACKGROUND OF THE INVENTION

Virus expression vectors allow rapid expression of host and non-host genes in plant systems. The construction of cDNA libraries within virus expression allows gene function to be assessed by screening plant hosts for phenotypic changes and gain
15 of function effects measured by various input and output focused screening. The ability of virus vectors to effectively reprogram host machinery to favor expression of virally encoded genes has been used to overexpress genes of pharmaceutical relevance in plants. This capacity has moved from being a mere laboratory curiosity, to being exploited at greenhouse and field scale to produce products used in human clinical
20 trials. These expression systems have primarily exploited dicot hosts, especially in the families of Solanaceae and Crucifereace. However, one report has been published for a potyvirus expression vector in monocot plants. This vector will allow the insertion of precise ORFs within the virus polyprotein for the accumulation of only cytosolically localized proteins (Choi, *et al.*, 2000). This type of vector is limited in
25 that only exact insertions are possible in this vector and not allowing insertion of crude cDNAs or libraries. It is further limited by not allowing the expression of secreted proteins in plants.

Continued research on virus expression vectors soon revealed new utilities. It has been extensively demonstrated that virus vectors are efficient tools to induce post-
30 transcriptional gene silencing systemically in host plants. However, these effects have only been demonstrated in dicot hosts, primarily in *Nicotiana benthamiana* and *Arabidopsis thaliana*. The power of shuttling in plant gene sequences systemically into plants and screening infected plants for loss of function effects is a unique tool to

determine function of endogenes. To confirm the effects of loss of function or gain of .function events discovered in dicot plants, the ability to either express genes or silence them in monocot system would provide a powerful tool to confirm gene function in a second system. However, at present, no such tool exists. It is the purpose of this

5   disclosure to describe the invention and reduction to practice of a monocot gene expression and gene silencing system.

Gopinath, *et al.* (2000) discloses a cowpea mosaic virus expressing a movement protein-green fluorescent protein ("GFP")-large coat protein fusion protein, with the foot and mouth disease virus 2A catalytic peptide at each side of the GFP

10   insert. However, expression of the fusion protein is only demonstrated in a cowpea plant, which is a dicot plant.

WO99/36516 disclose the use of viral vectors to silence endogenous genes in a host, through post-transcription gene silencing of the endogenous host gene homolog, by introducing nucleic acids into the host by way of constructs based on tobacco

15   mosaic virus or tomato mosaic virus.

Vectors for the genetic manipulation of plants have been derived from several naturally occurring plant viruses, including barley stripe mosaic virus ("BSMV"). BSMV is a tripartite RNA virus (Palomar, *et al.*, 1977) (Figure 1A) that infects many agriculturally important monocot species such as oat, wheat and barley (McKinney

20   and Greeley, 1965). We developed several monocot viral vectors based on the BSMV genome that direct the expression of nucleotide sequences in transfected protoplasts and plants.

SUMMARY OF THE INVENTION

25   The present invention is directed to recombinant viruses comprising a viral genome or one or more recombinant viral vectors, each further comprising a polynucleotide encoding a fusion protein or a foreign protein of interest or a targeting nucleotide sequence capable of silencing a gene of interest. The present invention is also directed to a host comprising the recombinant virus, recombinant viral vector, or

30   polynucleotide. The present is also directed to methods of expressing the fusion protein or a foreign protein, or silencing a gene of interest in a host.

(A) One aspect of the present invention is directed to one or more polynucleotide(s), wherein at least one of the one or more polynucleotide encodes a

promoter operatively linked to a transcriptional unit, wherein the transcriptional unit encodes a fusion protein, wherein the fusion protein comprises (1) a viral protein, (2) a protein of interest, and (3) autoproteolytic peptide, wherein the autoproteolytic peptide is fused between the viral protein and the protein of interest. In one

5 embodiment, the protein of interest is able to be directed to a specific location within the host cell.

(B) Another aspect of the present invention is also directed to one or more polynucleotide(s), wherein at least one of the one or more polynucleotide(s) encodes a promoter operatively linked to a transcriptional unit, wherein the transcriptional unit

10 encodes a protein of interest, wherein the one or more polynucleotide(s) constitute a viral genome, wherein the gene, or fragment thereof, encoding a coat protein of the viral genome is deleted or mutated, wherein the coat protein is native to the viral genome. The deletion or mutation of the coat protein gene, or fragment thereof, causes an increased, elevated, enhanced, or high expression of the transcription unit

15 when compared to a identical one or more polynucleotide(s) that does not have the deletion or mutation of the coat protein gene, or fragment thereof.

(C) Another aspect of the present invention is also directed to one or more polynucleotide(s), wherein at least one of the one or more polynucleotide(s) encodes a promoter operatively linked to a transcriptional unit, wherein the transcriptional unit

20 encodes a protein of interest, wherein said polynucleotide is a duplicated genomic nucleic acid component. In one embodiment of the invention, there is only one duplicated genomic nucleic acid component.

(D) Another aspect of the present invention is also directed to one or more polynucleotide(s), wherein at least one of the one or more polynucleotide(s) encodes a

25 promoter operatively linked to a transcriptional unit, wherein the transcriptional unit encodes a viral protein, a stop codon, and a targeting nucleotide sequence, wherein the a viral protein is 5' of the stop codon, and the stop codon is 5' of the targeting nucleotide sequence, wherein the viral protein, the stop codon, and the targeting nucleotide sequence are transcribed as a transcriptional unit, wherein the targeting

30 nucleotide sequence is substantially homologous to a gene of interest, or fragment thereof, to be silenced. The target nucleotide sequence can be in the sense or antisense orientation of the gene of interest to be silenced. Preferably the target

nucleotide sequence is in the sense orientation. Preferably silencing takes place in the cytoplasm.

BRIEF DESCRIPTION OF THE FIGURES

5   Figure 1A depicts the genetic structure of the BSMV γ42 clone.

Figure 1B depicts the genetic structure of three RNAγ constructs: "γ42.NheI", "γ42.NheI-B", and "γ42.NheI-S" (including the restriction sites for NheI, MluI, BssHII, and SwaI).

Figure 1C depicts the genetic structure of four RNAγ constructs:

10   "γ.γb.stop.P/N-zeo-B", "γ.γb.stop.N/P-zeo-B","γ.γb.stop.P/N-zeo-S", and "γ.γb.stop.N/P-zeo-S" (including the restriction sites for NheI, PacI, NotI, BssHII, and SwaI).

Figure 2A depicts the genetic structure of two RNAγ constructs: "γ.γb::GFPc3" and "γ.γb::GFPc3-as" (including the restriction sites for PacI and NotI).

15   Figure 2B depicts the genetic structure of a RNAγ construct: γ.GFPc3::γb (including the restriction sites for PacI and NotI).

Figure 2C depicts the genetic structure of two RNAγ constructs: "γ.γb::2A::GFPc3" and "γ.GFPc3::2A::γb" (including the restriction sites for PacI and NotI).

20   Figure 2D depicts the genetic structure of three RNAγ constructs: "γ.GFPc3::HA::2A::γb", "γ.GFPc3::myc::2A::γb", and "γ.GFPc3::H::2A::γb" (including the restriction sites for PacI and NotI).

Figure 2E depicts the genetic structure of three RNAγ constructs: "γ.Δγb.GFPc3", "γ.Δγb ", and "γ.GFPc3.stop.γb".

25   Figure 3A depicts the Western blot analysis of protein extracted from barley or *N. benthamiana* infected with recombinant BSMV or recombinant TMV probed with an anti-BMV coat protein antibody. Lane M contains the marker. Lane 1 contains protein extracted from barley infected with the α, βΔβa, γ.GFPc3::H::2A::γb recombinant BSMV. Lane 2 contains protein extracted from barley infected with the

30   α, βΔβa, γ.BMV-CP::H::2A::γb recombinant BSMV. Lane 3 contains protein extracted from *N. benthamiana* infected with the TMV SBS5::GFP recombinant

TMV. Lane 4 contains protein extracted from *N. benthamiana* infected with the TMV SBS5::BMV-CP recombinant TMV.

Figure 3B depicts the Western blot analysis of protein extracted from barley or *N. benthamiana* infected with recombinant BSMV or recombinant TMV probed with an anti-chicken interferon (chIF) antibody. Lane M contains the marker. Lane 1 contains protein extracted from barley infected with the α, βΔβa, γ.GFPc3::H::2A::γb recombinant BSMV. Lane 2 contains protein extracted from barley infected with the α, βΔβa, γ.chIF::H::2A::γb recombinant BSMV. Lane 3 contains protein extracted from *N. benthamiana* infected with the TMV SBS5::GFP recombinant TMV. Lane 4 contains protein extracted from *N. benthamiana* infected with the TMV SBS5::chIF recombinant TMV.

Figure 3C depicts the Western blot analysis of protein extracted from barley or *N. benthamiana* infected with recombinant BSMV or recombinant TMV probed with an anti-bovine lysozyme (boLys) antibody. Lane M contains the marker. Lane 1 contains protein extracted from barley infected with the α, βΔβa, γ.GFPc3::H::2A::γb recombinant BSMV. Lane 2 contains protein extracted from barley infected with the α, βΔβa, γ.boLys::H::2A::γb recombinant BSMV. Lane 3 contains protein extracted from *N. benthamiana* infected with the TMV SBS5::GFP recombinant TMV. Lane 4 contains protein extracted from *N. benthamiana* infected with the TMV SBS5::boLys recombinant TMV.

Figure 3D depicts the Western blot analysis of protein extracted from barley or *N. benthamiana* infected with recombinant BSMV or recombinant TMV probed with an anti-human growth hormone (hGH) antibody. Lane M contains the marker. Lane 1 contains protein extracted from barley infected with the α, βΔβa, γ.GFPc3::H::2A::γb recombinant BSMV. Lane 2 contains protein extracted from barley infected with the α, βΔβa, γ.hGH::H::2A::γb recombinant BSMV. Lane 3 contains protein extracted from *N. benthamiana* infected with the TMV SBS5::GFP recombinant TMV. Lane 4 contains protein extracted from *N. benthamiana* infected with the TMV SBS5::hGH recombinant TMV.

Figure 3E depicts the Western blot analysis of protein extracted from barley or *N. benthamiana* infected with recombinant BSMV or recombinant TMV probed with an anti-tomato protease inhibitor (tPI1) antibody. Lane M contains the marker. Lane

1 contains protein extracted from barley infected with the α, βΔβa, γ.GFPc3::H::2A::γb recombinant BSMV. Lane 2 contains protein extracted from barley infected with the α, βΔβa, γ.tPI1::H::2A::γb recombinant BSMV. Lane 3 contains protein extracted from N. benthamiana infected with the TMV SBS5::GFP

5   recombinant TMV. Lane 4 contains protein extracted from N. benthamiana infected with the TMV SBS5::tPI1 recombinant TMV.

Figure 4A depicts the genetic structure of two RNAβ constructs: "β.γb::βb" and "β.γb::2A::βb".

Figure 4B depicts the genetic structure of the RNAβ construct: "β.Δβa"

10   (including the restriction site for BstBI).

Figure 4C depicts the genetic structure of two RNAβ constructs: "β.GFPc3::βb" and "β.GFPc3::2A::βb".

Figure 4D depicts the genetic structure of the RNAβ construct: "β.GFPc3.Δβb" (including the restriction sites for EcoRI).

15   Figure 4E depicts the genetic structure of two RNAγ constructs: "γ.γb::βb" and "γ.γb::2A::βb".

Figure 5A depicts the genetic structure of the RNAγ construct: "γ.Δγa.Δγb.GFPc3" (including the restriction sites for EcoRV).

Figure 5B depicts the genetic structure of two RNAγ constructs: "γ.Δγb" and

20   "γ.Δγa" (including the restriction sites for EcoRV).

Figure 5C depicts the genetic structure of the RNAγ construct: "γ.Δγb.GFPc3".

Figure 5D depicts the Western blot analysis of protein extracted from tobacco protoplasts infected with recombinant BSMV or recombinant TMV probed with an anti-GFP antibody. Lane M contains the marker. Lane 1 contains protein extracted

25   from tobacco protoplasts infected with the TMV SBS5::GFP recombinant TMV (inoculation number 2, see Table 3). Lane 2 contains protein extracted from tobacco protoplasts infected with the α, β, γ.Δγa.Δγb.GFPc3 recombinant tobacco (inoculation number 5, see Table 3). Lane 3 contains protein extracted from tobacco protoplasts infected with the α, β, γ, γ.Δγa.Δγb.GFPc3 recombinant BSMV (inoculation number

30   6, see Table 3). Lane 4 contains protein extracted from tobacco protoplasts infected with the α, βΔβa, γ.Δγa.Δγb.GFPc3 recombinant BSMV (inoculation number 13, see

Table 3). Lane 5 contains protein extracted from tobacco protoplasts infected with the

α, βΔβa, γ.Δγa.Δγb.GFPc3 (2x) recombinant BSMV (inoculation number 17, see

Table 3).

Figure 5E depicts the genetic structure of two RNAγ constructs:

"γ.γb.stop.PDS Sense cDNA" and "γ.γb.stop.PDS Antisense cDNA " (including the

restriction sites for PacI and NotI).

Figure 6 depicts the HPLC analysis of phytoene accumulation in barley at 16

days post inoculation (dpi) inoculated with BSMV::GFP (leaf 5; green). The solid

line depicts the 285 nm absorption spectra and the dotted line depicts the 425 nm

absorption spectra.

Figure 7 depicts the HPLC analysis of phytoene accumulation in barley at 16

days post inoculation (dpi) inoculated with wild-type BSMV (leaf 5; green). The

solid line depicts the 285 nm absorption spectra and the dotted line depicts the 425 nm

absorption spectra.

Figure 8 depicts the HPLC analysis of phytoene accumulation in barley at 16

days post inoculation (dpi) inoculated with BSMV::bPDS (leaf 5; proximal to stem,

green). The solid line depicts the 285 nm absorption spectra and the dotted line

depicts the 425 nm absorption spectra.

Figure 9 depicts the HPLC analysis of phytoene accumulation in barley at 16

days post inoculation (dpi) inoculated with BSMV::bPDS (leaf 5; distal to stem, 75%

bleached). The solid line depicts the 285 nm absorption spectra and the dotted line

depicts the 425 nm absorption spectra.

Figure 10 depicts the HPLC analysis of phytoene accumulation in barley at 16

days post inoculation (dpi) inoculated with BSMV::bPDS (leaf 4; 25% bleached). The

solid line depicts the 285 nm absorption spectra and the dotted line depicts the 425 nm

absorption spectra.

Figure 11 depicts the HPLC analysis of phytoene accumulation in barley at 16

days post inoculation (dpi) treated with the chemical inhibitor of PDS, norflurazon

(leaf 3; green). The solid line depicts the 285 nm absorption spectra and the dotted

line depicts the 425 nm absorption spectra.

Figure 12 depicts the HPLC analysis of phytoene accumulation in barley at 16

days post inoculation (dpi) treated with the chemical inhibitor of PDS, norflurazon

(leaf 4; green). The solid line depicts the 285 nm absorption spectra and the dotted line depicts the 425 nm absorption spectra.

Figure 13 depicts the HPLC analysis of phytoene accumulation in barley at 16 days post inoculation (dpi) treated with the chemical inhibitor of PDS, norflurazon (leaf 5; completely bleached). The solid line depicts the 285 nm absorption spectra and the dotted line depicts the 425 nm absorption spectra.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to recombinant viruses comprising a viral genome or one or more recombinant viral vectors, each further comprising a polynucleotide encoding a fusion protein or a foreign protein of interest or a targeting nucleotide sequence capable of silencing a gene of interest. The present invention is also directed to a host comprising the recombinant virus, recombinant viral vector, or polynucleotide. The present is also directed to methods of expressing the fusion protein or a foreign protein, or silencing a gene of interest in a host.

(A) One aspect of the present invention is directed to one or more polynucleotide(s), wherein at least one of the one or more polynucleotide encodes a promoter operatively linked to a transcriptional unit, wherein the transcriptional unit encodes a fusion protein, wherein the fusion protein comprises (1) a viral protein, (2) a protein of interest, and (3) autoproteolytic peptide, wherein the autoproteolytic peptide is fused between the viral protein and the protein of interest. In one embodiment, the protein of interest is able to be directed to a specific location within the host cell. In one embodiment of the invention, the fusion protein consists of one viral protein, one protein of interest, and one or more autoproteolytic peptide. In another embodiment of the invention, the fusion protein consists of one viral protein, more than one protein of interest(s), and more than one autoproteolytic peptide(s). In another embodiment of the invention the polynucleotide(s) is derived from a multipartite virus. In another embodiment of the invention the polynucleotide(s) is derived from a hordeivirus. Preferably, the viral protein stay in the cytoplasm and the protein of interest is directed into a specific location within the host cell, such as an organelle, such as a chloroplast, an endoplasmic reticulum, an apoplast, or the like, or to be secreted out of the cell.

(B) Another aspect of the present invention is also directed to one or more polynucleotide(s), wherein at least one of the one or more polynucleotide(s) encodes a promoter operatively linked to a transcriptional unit, wherein the transcriptional unit encodes a protein of interest, wherein the one or more polynucleotide(s) constitute a viral genome, wherein the gene, or fragment thereof, encoding a coat protein of the viral genome is deleted or mutated, wherein the coat protein is native to the viral genome. The deletion or mutation of the coat protein gene, or fragment thereof, causes an increased, elevated, enhanced, or high expression of the transcription unit when compared to a identical one or more polynucleotide(s) that does not have the deletion or mutation of the coat protein gene, or fragment thereof. In one embodiment of the invention, the coat protein and the transcriptional unit are encoded on separate polynucleotides. In another embodiment of the invention, the coat protein is deleted and the promoter is the promoter of the γb gene of a barley stripe mosaic virus.

(C) Another aspect of the present invention is also directed to one or more polynucleotide(s), wherein at least one of the one or more polynucleotide(s) encodes a promoter operatively linked to a transcriptional unit, wherein the transcriptional unit encodes a protein of interest, wherein said polynucleotide is a duplicated genomic nucleic acid component. In one embodiment of the invention, there is only one duplicated genomic nucleic acid component. In another embodiment of the invention, there is more than one duplicated genomic nucleic acid component. In another embodiment of the invention, where there is more than one duplicated genomic nucleic acid component, all the duplicated genomic nucleic acid components encoded the identical transcription unit. In another embodiment of the invention, where there is more than one duplicated genomic nucleic acid component, at least two duplicated genomic nucleic acid components encode two different transcription units.

(D) Another aspect of the present invention is also directed to one or more polynucleotide(s), wherein at least one of the one or more polynucleotide(s) encodes a promoter operatively linked to a transcriptional unit, wherein the transcriptional unit encodes a viral protein, a stop codon, and a targeting nucleotide sequence, wherein the a viral protein is 5' of the stop codon, and the stop codon is 5' of the targeting nucleotide sequence, wherein the viral protein, the stop codon, and the targeting nucleotide sequence are transcribed as a transcriptional unit, wherein the targeting nucleotide sequence is substantially homologous to a gene of interest, or fragment

thereof, to be silenced. The target nucleotide sequence can be in the sense or antisense orientation of the gene of interest to be silenced. In one embodiment, the targeting nucleotide sequence is in the sense orientation and the silencing of the gene of interest takes place in the cytoplasm. In one embodiment of the invention, the

5   targeting nucleotide sequence comprises the nucleotide sequence of a gene of a first species of organism, and the gene of interest to be silenced is a gene of a second species of organism. In another embodiment, the gene of interest to be silenced is known. In another embodiment, the gene of interest to be silenced is unknown. In another embodiment of the invention, there is no viral protein encoded by the

10   transcriptional unit, the viral protein native to the promoter may be expressed from a different operon.

        Different aspects of the invention, (A) to (D), may be combined. For example, (A) can be combined with (B) to form an embodiment of the invention whereby a deletion of the coat protein causes an increased expression of a fusion protein; the

15   increased expression of which in turn causes a greater level of production of the protein of interest. For example, (A) can be combined with (C) to form an embodiment of the invention whereby a transcriptional unit encoding a fusion protein is present on a plurality of duplicate genomic nucleic acid components, causing an increased expression of the protein of interest. For example, (B) can be combined

20   with (C) to form an embodiment of the invention whereby a deletion of the coat protein causes an increased expression of a transcriptional unit encoding a protein of interest on a duplicated genomic nucleic acid component, causing an increased expression of the protein of interest. For example, (B) can be combined with (D) to form an embodiment of the invention whereby a deletion of the coat protein causes an

25   increased expression of a transcriptional unit encoding a targeting nucleotide sequence; the increased expression of which in turn causes a greater effect in the silencing of a gene of interest. For example, (C) can be combined with (D) to form an embodiment of the invention whereby a transcriptional unit encoding a targeting nucleotide sequence is present on a plurality of duplicate genomic nucleic acid

30   components; the increased expression of which in turn causes a greater effect in the silencing of a gene of interest. For example, (B), (C), and (D) can be combined together to form an embodiment of the invention whereby a transcriptional unit encoding a targeting nucleotide sequence is present on a plurality of duplicate

genomic nucleic acid components, and the coat protein is deleted thereby causing a further increased expression of the transcriptional unit; the increased expression of which in turn causes a greater effect in the silencing of a gene of interest. These examples serve to exemplify and do not limit the possible combinations possible.

5   The polynucleotide may be a nucleic acid, a recombinant nucleic acid, a recombinant viral nucleic acid, a genomic nucleic acid component, a recombinant polynucleotide, or the like. The polynucleotide may be DNA or RNA, either double-strand or single strand. Single-strand DNA or RNA can be either positive- or plus-sense, or negative- or minus-sense. The polynucleotide may also contain synthetic

10   nucleic acid or nucleotides in the stead of DNA or RNA. The polynucleotide may be part of a vector, construct, plasmid, episome, replicon, or the like. The polynucleotide may be viral and may be recombinant or both. The polynucleotide may be a viral expression vector or a recombinant viral expression vector. The polynucleotide can remain extra-chromosomal and need not integrate into any host chromosome. The

15   polynucleotide can be maintained in the cytoplasm of the host and does not need to enter the nucleus of the host and is able to replicate in the cytoplasm of the host. The genomic nucleic acid component may be a subgenomic nucleic acid. For example, if the genome is that of a hordeivirus, such as a BSMV, then the subgenomic nucleic acid may be RNAα, RNAβ, or RNAγ. The genomic nucleic acid component may also

20   be a duplicated subgenomic nucleic acid. The polynucleotide may or may not be encapsidated by coat protein(s) encoded by the recombinant virus. The recombinant viral nucleic acid may or may not have individual features common to certain viruses, such as a cap at the 5' terminus of the nucleic acid, a specific initial sequence, or a highly conserved 3' terminus of the nucleic acid. A cap may be a 7-methylguanosine

25   cap. A specific initial sequence may be an initial sequence of $m^7GpppGUA$. A highly conserved 3' terminus may be a polyadenylate (poly A) sequence that separates the coding region from a 238 nucleotide 3' terminal tRNA-like structure. The tRNA-like structure may be able to be aminoacylated with tyrosine. The recombinant viral nucleic acid or recombinant virus is used to infect a host. The recombinant nucleic

30   acid is capable of replication in the host, localized or systemic spread in the host, and transcription or expression of the native nucleic acid in the host to affect a phenotypic or biochemical change in the host.

The transcriptional unit is any sequence of DNA or RNA that is able to be transcribed into a mRNA, message, transcript, transcriptional product, or the like. The transcriptional unit may encode one or more ORFs that may or may not be translated into an amino acid sequence that constitutes a peptide or protein. The

5   transcription may take place in the host or *in vivo* or *in vitro*. The transcription unit may also encode or contain or comprise or consist one or more targeting nucleotide sequence. The invention provides for the expression of the transcriptional unit in the cytoplasm of the host.

The viral protein is any protein of any virus. The viral protein may be native

10   or non-native or heterologous to the recombinant viral vector that comprises the polynucleotide, and/or the recombinant virus that comprises the recombinant viral vector. The viral protein may be a polyprotein or a non-polyprotein. The viral protein can be any viral protein that does not interfere in the expression of the transcriptional unit or fusion protein, if present, or the cleavage of the autoproteolytic peptide, if

15   present. In a preferred embodiment the viral protein is one that is expressed at a high level relative to the other proteins expressed from the recombinant virus, recombinant vector, or polynucleotide.

The protein of interest can be a peptide of virtually any amino acid sequence as long as the expression of the protein of interest does not interfere in the expression

20   of the transcriptional unit or fusion protein, if present, or the cleavage of the autoproteolytic peptide, if present. The protein of interest may vary in size from one amino acid residue to several hundred amino acid residues. The protein of interest may be a green fluorescent protein or any protein that is not green fluorescent protein. The protein of interest may be any protein one desires to be expressed in a host in

25   order for the purpose of causing a phenotypic or biochemical change of the host. The phenotypic or biochemical change may be involved or related to metabolism, host structure, rate of growth, pathogen or pest resistance, resistance to environmental conditions, growth requirements, propagation, pigmentation, or the like. Alternatively, the protein of interest may be any protein one desires to be expressed in

30   a host in order for the purpose of harvesting or purifying the protein of interest from the host or products produced by the host. The protein of interest can be synthesized and directed into any specific location of the host cell or secreted out of the cell. Preferably the viral protein stays in the cytoplasm. The protein of interest can be

synthesized and directed into any specific location of the host cell, such as an organelle, the following are such an organelle: nucleus, nucleolus, chloroplast, vacuole, pinocytic vesicle, rough endoplasmic recticulum, smooth endoplasmic recticulum, Golgi body, apoplast, or the like. Preferably the protein of interest

5   comprises a signal peptide that can direct the protein of interest to be localized to a specific location within the host cell, such as an organelle. Preferably the signal peptide (SP) is at the N-terminal of the protein of interest. Preferably the protein of interest is of a suitable size or number of amino acids as exemplified by the size and number of amino acids of bovine lysozyme, chicken interferon, human growth

10   hormone, and protease inhibitor.

The autoproteolytic peptide is any amino acid sequence that can essentially independently cleave itself by breaking a peptide bond or covalent bond with its sequence in the presence of heterologous amino acid sequences at both its N- and C-terminal ends. An example of an amino acid sequence that essentially contains an

15   autoproteolytic cleavage activity is the 2A peptide of the foot and mouth disease virus ("FMDV"). Examples of these are: NFDLLKLAGDVESNPGP (SEQ ID NO: 1) (Mattion et al., J. Virol. 70(11):8124-7 (1996)), QLLNFDLLKLAGDVESNLGP (SEQ ID NO: 2) (FMD-A10), QLLNFDLLKLAGDVESNPRP (SEQ ID NO: 3) (FMD-A12), QLLNFDLLKLAGDVESNPGP (SEQ ID NO: 4) (FMD-C1),

20   QTLNFDLLKLAGDVESNPGP (SEQ ID NO: 5) (FMD-O1k), QLLNFDLLKLAGDVESNPGP (SEQ ID NO: 6) (FMD-Sat2), HYAGYFADLLIHDIETNPGP (SEQ ID NO: 7) (EMC-R), HYAGYFSDLLIHDVETNPGP (SEQ ID NO: 8) (Mengo), YHADYYKQRLIHDVEMNPGP (SEQ ID NO: 9) (TME-DA) (Palmenberg, 1990).

25   The 2A peptide cleavage occurs at the C-terminal end of the glycine-proline amino acid pair. For example, the autoproteolytic peptide QLLNFDLLKLAGDVESNPGP (SEQ ID NO: 6) is cleaved into two separate portions: QLLNFDLLKLAGDVESNPG (SEQ ID NO: 10) and a proline. The former portion is attached to the C-terminus of whatever peptide was attached to the N-

30   terminus of the autoproteolytic peptide, and the proline is attached to the N-terminus of whatever peptide was attached to the C-terminus of the autoproteolytic peptide. The autoproteolytic peptide may be cleaved prior to the complete translation of the ORF encoding the entire fusion protein. The autoproteolytic peptide preferably

contains no more than twenty amino acid residues. More preferably, the autoproteolytic peptide contains no more than seventeen amino acid residues. The autoproteolytic peptide preferably after cleavage does not leave sufficient residual amino acid residues attached to the viral protein that will interfere with the

5   biochemical or biological activity or function of the viral protein, especially in the ability of the virus to replicate, assemble, move from cell to cell, achieve systemic infection, etc. The autoproteolytic peptide preferably after cleavage does not leave sufficient residual amino acid residues attached to protein of interest that will interfere with the biochemical or biological activity or function of the protein of interest in its

10   effect on the host or purification or any characteristic of the protein of interest that is of interest to the practitioner of the invention. In a preferred embodiment the peptide that is subject to cleavage is the 2A peptide, or a fragment thereof that essentially contains an autoproteolytic cleavage activity, of a FMDV. The autoproteolytic peptide may be linked to the N- or C-terminal end of the viral protein. If the

15   autoproteolytic peptide is linked to the N-terminal end of the viral protein, then the protein of interest is linked to the N-terminal end of the autoproteolytic peptide. If the autoproteolytic peptide is linked to the C-terminal end of the viral protein, then the protein of interest is linked to the C-terminal end of the autoproteolytic peptide. These links may comprise a covalent bond such as a peptide bond. One of ordinary

20   skill in the art is able to construct a fusion protein comprising an autoproteolytic peptide (DeFelipe, et al., 2000; Gopinath, et al., 2000; Kokuho, et al., 1999; DeFilipe, et al., 1999; Chaplin, et al., 1999; Halpin, et al., 1999; Donnelly, et al., 1997; Mattion, et al., 1996; Li, et al., 1996; Ryan, et al., 1994; Ryan, et al., 1991).

The promoter is operatively linked to the transcriptional unit so that the

25   transcriptional unit is capable of being expressed in the host. The promoter is linked 5' to transcriptional unit. Any native or non-native promoter may be used. The promoter may also be native or non-native to the viral protein. The expression of the transcriptional unit may be driven by any of a variety of promoters functional in the genome of the recombinant virus in the host. The transcriptional unit may also be

30   expressed by any promoter functional in a host, that is a plant or a plant cell, 5' to the transcriptional unit. In a preferred embodiment, the host is a plant cell, a plant protoplast, a cell in a plant cell culture, or any appropriate cell. The promoter may be any viral promoter or RNA viral promoter or viral subgenomic promoter. In a

preferred embodiment, the promoter is any promoter of a RNA plant virus. In a more preferred embodiment, the promoter is any promoter of a single-stranded plus-sense RNA plant virus. In an even more preferred embodiment, the promoter is any promoter of a multipartite single-stranded plus-sense RNA plant virus. In an even

5 further more preferred embodiment, the promoter is any promoter of a tripartite single-stranded plus-sense RNA plant virus, such as a hordeivirus. In an even much further more preferred embodiment, the promoter is any promoter of BSMV, such as the promoters of the βb or γb gene. In another preferred embodiment of the invention, the fusion proteins are expressed from plant viral subgenomic promoters using vectors

10 as described in U.S. Patent 5,316,931. The promoter need not be a naturally occurring promoter but can also be a synthetic promoter designed to initiate transcription of the transcriptional unit in a host. The expression of the fusion protein may be elevated or controlled by a variety of plant or viral transcription factors. In addition, there is no need to use a promoter that is an additional subgenomic promoter.

15 The promoter may also be a plant promoter. The plant promoter may be a tissue- or developmentally-regulated and/or an inducible promoter. Inducible promoters may be advantageous in certain circumstances because they place the timing of expression of the transcriptional unit under the control of the user. The term "inducible" as applied to a promoter is well understood by those of ordinary skill in

20 the art. In essence, expression under the control of an inducible promoter is "switched on" or increased in response to an applied stimulus. The nature of the stimulus varies between promoters. Some inducible promoters cause little or undetectable levels of expression (or no expression) in the absence of the appropriate stimulus. Other inducible promoters cause detectable constitutive expression in the absence of the

25 stimulus. Whatever the level of expression is in the absence of the stimulus, expression from any inducible promoter is increased in the presence of the correct stimulus. The preferable situation is where the level of expression increases upon application of the relevant stimulus by an amount effective to alter a phenotypic or biochemical change. Thus an inducible (or "switchable") promoter may be used

30 which causes a basic level of expression in the absence of the stimulus which level is too low to bring about a desired phenotype (and may in fact be zero). Upon application of the stimulus, expression is increased (or "switched on") to a level which brings about the desired phenotypic or biochemical change.

The expression of the transcriptional unit, fusion protein, protein of interest, or viral protein of the subject invention can take place *in vitro*, *in vivo*, or in the cytoplasm of the host. The targeting nucleotide sequence comprises a nucleotide sequence that is identical or complementary to a gene of interest to be silenced, either in the sense or antisense orientation, or a sequence which has sufficient homology to a gene of interest to be silenced for down-regulation of the expression of the target sequence to occur. The targeting nucleotide sequence in its entirety may or may not encode an ORF. The targeting nucleotide sequence may have the identical or complementary sequence of the gene of interest to be silenced. The targeting nucleotide sequence may be able to silence one or more genes; in this case, there is more than one gene of interest to be silenced.

The gene of interest to be silenced can be any gene found in the host. The gene of interest may code for a rRNA, tRNA, ribozyme, or any peptide or protein. The targeting nucleotide sequence can be a stretch of nucleotides of any length as long the length or sequence does not interfere with the expression of the transcriptional unit or the silencing of the gene of interest. The targeting nucleotide sequence can be as short as 200 nucleotides. The targeting nucleotide sequence may contain the ORF of one gene of organism, and be able silence the gene of that organism, and/or one or more different gene(s) of that organism, and/or one or more different gene(s) of one or more different organism(s). For example, a targeting nucleotide sequence comprising a fragment of the ORF of phytoene desaturase (PDS) of rice (*Oryza sativa*) or corn (*Zea mays*), when expressed in barley, silences the expression of PDS of barley (see Example 9). The targeting nucleotide sequence is not able to be translated from the transcriptional unit. This is because there is one or more stop codon(s) adjacent 5' to or immediately preceding the region of sequence encoding the protein, or fragment thereof. The targeting nucleotide sequence, if containing one or more ORF(s), need not be in frame with any translatable regions of nucleotide sequence 5' of the targeting nucleotide sequence. The transcriptional unit may comprise a plurality of one targeting nucleotide sequence, different targeting nucleotide sequences, or a combination of the same or different targeting nucleotide sequences. Different targeting nucleotide sequence may silence the same gene of interest to be silenced. One targeting nucleotide sequence may silence a plurality of genes of interest to be

silenced. There may be one gene of interest to be silenced in a host, or there may be a plurality of genes of interest to be silenced in a host.

The term "silence" or "silenced" denotes a lowered or decreased expression of a gene. Any degree of lowering or decreasing of the expression of a gene is sufficient to constitute the gene to be silenced. The degree of lowering or decreasing of the expression of a gene may be of a degree sufficient to detect a phenotypic or biochemical of a host comprising the gene. The gene to be silenced may only be expressed in a host temporally or spatially within an organism, in such cases silencing would only occur when the gene to be silenced in the host at a time and/or place when the gene would have been expressed. The user of the invention may cause silencing to take place only in a temporal- and/or spatially-specific manner. Within each host cell, silencing preferably takes place within the cytoplasm. More preferably, silencing takes place in the cytoplasm of a plant cell. Even more preferably, silencing takes place in the cytoplasm of a monocot plant cell.

Silencing one or more gene(s) of interest may produce one or more results desirable to a user. For example, it may be desirable to silence a gene in a commercial fruit or vegetable that causes faster ripening in order to improve the shelf life of the fruit or vegetable. For example, it may be desirable to silence a gene in a commercial fruit or vegetable that causes faster ripening in order to improve processing and handling characteristics of the fruit or vegetable and to lengthen the shelf life of the fruit or vegetable. For example, it may be desirable to silence a gene involved in pollen formation of a commercial plant in order to that breeders can reproducibly generate male sterile plants for the production of F1 hybrids. For example, it may be desirable to silence a gene involved in lignin formation in order to facilitate paper manufacturing from vegetative tissues of the plant. For example, it may be desirable to silence a gene involved in pigment production in order to produce novel color or color patterns of flowers of ornamental plants. For example, it may be desirable to silence a gene involved in regulatory pathways controlling development or environmental responses to produce plants with novel growth habit or disease/pest resistance. For example, it may be desirable to silence a gene involved in production of toxins in order to eliminate the production of toxic secondary metabolites. For example, it may be desirable to silence a gene encoding an enzyme in a metabolic

pathway of a commercial fruit or vegetable plant in order to cause an increase of the level of an intermediate metabolic product in the fruit or vegetable.

The one or more polynucleotide(s) of the present invention can be part of a recombinant virus. The recombinant virus may be a DNA or RNA virus. The
5   recombinant virus can be derived from a plant virus. In a preferred embodiment, the virus is a RNA plant virus. In a more preferred embodiment, the virus is a single-stranded plus-sense RNA plant virus. In an even more preferred embodiment, the virus is a multipartite single-stranded plus-sense RNA plant virus. In an even further more preferred embodiment, the virus is a tripartite single-stranded plus-sense RNA
10   plant virus, such as a hordeivirus. In an even much further more preferred embodiment, the virus is a BSMV. Preferred viruses have the following characteristics: (a) ability to generate a high level of transcription of the ORF encoding the fusion protein in the host, (b) ability to tolerate the ORFs of the fusion protein in the life cycle of the virus, including replication, assembly, viral movement,
15   systemic infection, etc., (c) the promoter must be able to tolerate the expression of fusion protein(s), and, (d) the viral genome only has to stay in the cytoplasm of the host, i.e., the viral genome neither has to be present in the nucleus nor integrated in a host chromosome. The one or more polynucleotide(s) of the present invention, when assembled as a recombinant virus, are preferably still infectious and/or capable of
20   systemic infection.

The construction of recombinant viruses or recombinant viral vectors may use a variety of methods known in the art. In preferred embodiments of the instant invention, the recombinant viruses and recombinant viral vectors are derived from RNA plant viruses. A variety of plant virus families may be used, such as
25   Bromoviridae, Bunyaviridae, Comoviridae, Geminiviridae, Potyviridae, and Tombusviridae, among others. Within the plant virus families, various genera of viruses may be suitable for the instant invention, such as alfamovirus, ilarvirus, bromovirus, cucumovirus, tospovirus, carlavirus, caulimovirus, closterovirus, comovirus, nepovirus, dianthovirus, furovirus, hordeivirus, luteovirus, necrovirus,
30   potexvirus, potyvirus, rymovirus, bymovirus, oryzavirus, sobemovirus, tobamovirus, tobravirus, carmovirus, tombusvirus, tymovirus, umbravirusa, and among others.

Within the genera of plant viruses, many species are particular preferred. They include alfalfa mosaic virus, tobacco streak virus, brome mosaic virus, broad

bean mottle virus, cowpea chlorotic mottle virus, cucumber mosaic virus, tomato spotted wilt virus, carnation latent virus, cauliflower mosaic virus, beet yellows virus, cowpea mosaic virus, tobacco ringspot virus, carnation ringspot virus, soil-borne wheat mosaic virus, tomato golden mosaic virus, cassava latent virus, barley stripe

5   mosaic virus, barley yellow dwarf virus, tobacco necrosis virus, tobacco etch virus, potato virus X, potato virus Y, rice necrosis virus, ryegrass mosaic virus, barley yellow mosaic virus, rice ragged stunt virus, Southern bean mosaic virus, tobacco mosaic virus, ribgrass mosaic virus, cucumber green mottle mosaic virus watermelon strain, oat mosaic virus, tobacco rattle virus, carnation mottle virus, tomato bushy

10   stunt virus, turnip yellow mosaic virus, carrot mottle virus, among others. In addition, RNA satellite viruses, such as tobacco necrosis satellite may also be employed.

A given plant virus may contain either DNA or RNA, which may be either single- or double-stranded. One example of plant viruses containing double-stranded DNA includes, but not limited to, caulimoviruses such as cauliflower mosaic virus

15   (CaMV). Representative plant viruses that contain single-stranded DNA are cassava latent virus, bean golden mosaic virus (BGMV), and chloris striate mosaic virus. Rice dwarf virus and wound tumor virus are examples of double-stranded RNA plant viruses. Single-stranded RNA plant viruses include tobacco mosaic virus (TMV), turnip yellow mosaic virus (TYMV), rice necrosis virus (RNV), brome mosaic virus

20   (BMV), and BSMV. The single-stranded RNA viruses can be further divided into plus-sense (or positive-stranded), minus-sense (or negative-stranded), or ambisense viruses. The genomic RNA of a plus sense RNA virus is messenger sense, which makes the naked RNA infectious. Many plant viruses belong to the family of plus sense RNA viruses. They include, for example, TMV, BMV, and others. RNA plant

25   viruses typically encode several common proteins, such as replicase/polymerase proteins essential for viral replication and mRNA synthesis, coat proteins providing protective shells for the extracellular passage, and other proteins required for the cell-to-cell movement, systemic infection and self-assembly of viruses. The plant virus may also comprises a genome of only one nucleic acid or a bipartite, tripartite or

30   multipartite genome. For general information concerning plant viruses, see Matthews, *Plant Virology*, 3rd Ed., Academic Press, San Diego (1991).

Selected groups of suitable plant viruses are characterized below. However, the invention should not be construed as limited to using these particular viruses, but

rather the method of the present invention is contemplated to include all plant viruses at a minimum. However, the invention should not be construed as limited to using these particular viruses, but rather the present invention is contemplated to include all suitable viruses. Some suitable viruses are characterized below.

5

TOBAMOVIRUS GROUP

The tobacco mosaic virus (TMV) is able to express genes at high levels in plants. TMV is a member of the tobamovirus group. The TMV virion is a tubular filament, and comprises coat protein sub-units arranged in a single right-handed helix

10   with the single-stranded RNA intercalated between the turns of the helix. TMV infects tobacco as well as other plants. TMV virions are 300 nm x 18 nm tubes with a 4 nm-diameter hollow canal, and consist of 2140 units of a single structural protein helically wound around a single RNA molecule. The genome is a 6395 base plus-sense RNA. The 5'-end is capped and the 3'-end contains a series of pseudoknots and

15   a tRNA-like structure that will specifically accept histidine. The genomic RNA functions as mRNA for the production of proteins involved in viral replication: a 126-kDa protein that initiates 68 nucleotides from the 5'-terminus, and a 183-kDa protein synthesized by readthrough of an amber termination codon approximately 10% of the time. Only the 183-kDa and 126-kDa viral proteins are required for the TMV

20   replication in trans. (Ogawa *et al., Virol.* 185:580-584 (1991)). Additional proteins are translated from subgenomic size mRNA produced during replication (Dawson, *Adv. Virus Res.,* 38:307-342 (1990)). The 30-kDa protein is required for cell-to-cell movement; the 17.5-kDa capsid protein is the single viral structural protein. The function of the predicted 54-kDa protein is unknown.

25   TMV assembly apparently occurs in plant cell cytoplasm, although it has been suggested that some TMV assembly may occur in chloroplasts since transcripts of ctDNA have been detected in purified TMV virions. Initiation of TMV assembly occurs by interaction between ring-shaped aggregates ("discs") of coat protein (each disc consisting of two layers of 17 subunits) and a unique internal nucleation site in

30   the RNA; a hairpin region about 900 nucleotides from the 3'-end in the common strain of TMV. Any RNA, including subgenomic RNAs containing this site, may be packaged into virions. The discs apparently assume a helical form on interaction with

the RNA, and assembly (elongation) then proceeds in both directions (but much more rapidly in the 3'- to 5'- direction from the nucleation site).

Another member of the Tobamoviruses, the Cucumber Green Mottle Mosaic virus watermelon strain (CGMMV-W) is related to the cucumber virus. Nozu *et al.*,

5   *Virol.* 45:577 (1971). The coat protein of CGMMV-W interacts with RNA of both TMV and CGMMV to assemble viral particles *in vitro*. Kurisu *et al.*, *Virol.* 70:214 (1976).

Several strains of the tobamovirus group are divided into two subgroups, on the basis of the location of the assembly of origin. Subgroup I, which includes the

10   vulgare, OM, and tomato strain, has an origin of assembly about 800-1000 nucleotides from the 3'-end of the RNA genome, and outside the coat protein cistron. Lebeurier *et al., Proc. Natl. Acad. Sci. USA* 74:149 (1977); and Fukuda *et al., Virology* 101:493 (1980). Subgroup II, which includes CGMMV-W and cornpea strain (Cc) has an origin of assembly about 300-500 nucleotides from the 3'-end of the RNA genome

15   and within the coat-protein cistron. The coat protein cistron of CGMMV-W is located at nucleotides 176-661 from the 3'-end. The 3' noncoding region is 175 nucleotides long. The origin of assembly is positioned within the coat protein cistron. Meshi *et al., Virology* 127:54 (1983).

20   BROME MOSAIC VIRUS GROUP

Brome Mosaic virus (BMV) is a member of a group of tripartite, single-stranded, RNA-containing plant viruses commonly referred to as the bromoviruses. Each member of the bromoviruses infects a narrow range of plants. Mechanical transmission of bromoviruses occurs readily, and some members are transmitted by

25   beetles. In addition to BV, other bromoviruses include broad bean mottle virus and cowpea chlorotic mottle virus.

Typically, a bromovirus virion is icosahedral, with a diameter of about 26 μm, containing a single species of coat protein. The bromovirus genome has three molecules of linear, positive-sense, single-stranded RNA, and the coat protein mRNA

30   is also encapsidated. The RNAs each have a capped 5'-end, and a tRNA-like structure (which accepts tyrosine) at the 3'-end. Virus assembly occurs in the cytoplasm. The complete nucleotide sequence of BMV has been identified and characterized as described by Ahlquist *et al., J. Mol. Biol.* 153:23 (1981).

RICE NECROSIS VIRUS

Rice Necrosis virus is a member of the Potato Virus Y Group or Potyviruses. The Rice Necrosis virion is a flexuous filament comprising one type of coat protein

5   (molecular weight about 32,000 to about 36,000) and one molecule of linear positive-sense single-stranded RNA. The Rice Necrosis virus is transmitted by *Polymyxa oraminis* (a eukaryotic intracellular parasite found in plants, algae and fungi).

GEMINIVIRUSES

10   Geminiviruses are a group of small, single-stranded DNA-containing plant viruses with virions of unique morphology. Each virion consists of a pair of isometric particles (incomplete icosahedral), composed of a single type of protein (with a molecular weight of about 2.7-3.4 x 10^4). Each geminivirus virion contains one molecule of circular, positive-sense, single-stranded DNA. In some geminiviruses

15   (i.e., Cassava latent virus and bean golden mosaic virus) the genome appears to be bipartite, containing two single-stranded DNA molecules.

POTYVIRUSES

Potyviruses are a group of plant viruses which produce polyprotein. A

20   particularly preferred potyvirus is tobacco etch virus (TEV). TEV is a well characterized potyvirus and contains a positive-strand RNA genome of 9.5 kilobases encoding for a single, large polyprotein that is processed by three virus-specific proteinases. The nuclear inclusion protein "a" proteinase is involved in the maturation of several replication-associated proteins and capsid protein. The helper

25   component-proteinase (HC-Pro) and 35-kDa proteinase both catalyze cleavage only at their respective C-termini. The proteolytic domain in each of these proteins is located near the C-terminus. The 35-kDa proteinase and HC-Pro derive from the N-terminal region of the TEV polyprotein.

30   ## HORDEIVIRUS GROUP

Hordeiviruses are a group of single-stranded, positive sense RNA-containing plant viruses with three or four part genomes. Hordeiviruses have rigid, rod-shaped virions. Hordeivirus is composed of four members: barley stripe mosaic virus

("BSMV"), poa semilatent virus ("PSLV"), lychnis ringspot virus ("LRSV"), and anthoxanthum latent blanching virus ("ALBV") (Jackson, *et al.*, 1989). BSMV is the type member of this group of viruses. BSMV infects a large number of monocot and dicot species including barley, oat, wheat, corn, rice, , spinach, and *Nicotiana*

5   *benthamiana.* Local lesion hosts include *Chenopodium amaranticolor,* and *Nicotiana tabacum* ccv. Samsun . BSMV is not vector transmitted but is mechanically transmissable and in some hosts, such as barley, is also transmitted through pollen and seed.

Most strains of BSMV have three genomic RNAs referred to as RNAα (or

10   αRNA), RNAβ (or βRNA), and RNAγ (or γRNA). At least one strain, the Argentina mild (AM) strain has a fourth genomic RNA that is essentially a deletion mutant of the RNAγ. All genomic RNAs are capped at the 5' end and have tRNA-like structures at the 3' end. Virus replication and assembly occurs in the cytoplasm. The complete nucleotide sequence of several strains of BSMV has been identified and characterized

15   (reviewed by Jackson, *et al.,* 1989), and infectious cDNA clones are available (Petty, *et al. Virol.* 171:342-349 (1989)).

BSMV is a plus-sense single-stranded RNA virus that is able to infect plants of the *Chenopodiaceae, Gramineae,* and *Solanaceae* families, including, but not limited to, the following species: *Anthoxanthum aristatum, Anthoxanthum odoratum,*

20   *Avena sativa, Beta vulgaris, Bromus secalinus, Bromus tectorum, Chenopodium album, Chenopodium amaranticolor, Chenopodium quinoa, Dactylis glomerata, Echinochloa crus-galli, Elytrigia intermedia, Eragrostis cilianensis, Festuca pratensis, Hordeum vulgare, Lagurus ovatus, Lolium multiflorum, Lolium perenne, Lolium persicum, Lolium temulentum, Lophopyrum elongatum, Nicotiana tabacum,*

25   *Oryza sativa, Oryzopsis miliacea, Panicum capillare, Panicum miliaceum, Phalaris arundinacea, Phalaris paradoxa, Phleum arenarium, Phleum pratense, Poa annua, Poa pratensis, Secale cereale, Setaria italica, Setaria macrostachya, Setaria viridis, Sorghum bicolor, Spinacia oleracea, Triticum aestivum, Triticum durum,* and *Zea mays.* The natural host range is *H. vulgare* and *T. aestivum.* The method of

30   transmission does not involve a vector and is by mechanical inoculation by seed (up to 90-100%) and by pollen to the pollinated plant. The morphology of the BSMV virions are rod-shaped, not enveloped, usually straight, with a clear modal length, of

112-150 nm, 18-24 nm wide, with an obvious axial canal (3-4 nm in diameter), and with a basic helix wiht a pitch of 2.5-2.6 nm. (Brunt *et al.*, 1996).

The BSMV virion contains 3.8-4% nucleic acid, 96% protein, and 0% lipid by weight. The BSMV genome consists of three single-stranded linear RNA (designated RNAα, RNAβ, and RNAγ). The total genome size is 10.289 kb (Brunt *et al.*, 1996). Each genomic RNA has a 7-methylguanosine cap at its 5' terminus and contains the initial sequence m^7GpppGUA, and has a highly conserved 3' terminus that has a polyadenylate (poly A) sequence that separates the coding region of each RNA from a 238 nucleotide 3' terminal tRNA-like structure that can be aminoacylated with tyrosine. BSMV encodes a total of seven polypeptides. RNAα encodes αa, a 130 kDa protein which is believed to be an integral component of viral replicase. αa has a putative methyltransferase domain near the N-terminus and a nucleotide binding motif near the C-terminus (Jackson *et al.*, 1991). When αa of BSMV strain N18 (non-pathogenic to oat) had more than half of its ORF replaced with the homologous αa of BSMV strain CV42 (pathogenic to oat), the gene homologous gene replacement enabled strain N18 to infect oat. In addition, a single amino acid substitution or up to six single amino acid substitutions (including the substitution of two adjacent amino acids) in αa of strain N18 enabled strain N18 to infect oat (Weiland *et al.*, 1996). RNAβ encodes four polypeptides: βa, the 22 kDa coat protein; βb, a 60 kDa disease-specific protein, which contains a nucleotide binding motif similar to αa; βc, a 17 kDa protein of unknown function but which is required for infectivity in barley (*N. benthamiana* and *C. amaranticolor*); and, βd, a 14 kDa protein essential for systemic infection and associated with the membrane fraction of infected barley. The ORFs of βb, βc and βd are tightly organized to form a triple gene block ("TGB") whereby βd overlaps βb and βc. The TGB is similar in organization to the overlapping gene blocks found in furoviruses, potexviruses, and potato virus M, a carlavirus (Jackson *et al.*, 1991). RNAγ encodes two ORFs: γa and γb. The γa ORF encodes a second replicase component, γa, that contains the GDD polymerase motif that is universally present in the replicases of plus-sense RNA viruses. The γb ORF encodes a 17 kDa cysteine rich protein, γb, contains a cysteine-rich region. BSMV with mutations which introduce single or up to four single amino acid substitutions in γb, when used to inoculate barley plants, resulted in altered symptom phenotype (Donald *et al.*,

1994). BSMV is of interest to provide new and improved vectors for the genetic manipulation of plants.

Genomic RNAα serves as an mRNA for the expression of the αa protein. Genomic RNAβ only serves as an mRNA for the expression of the βa protein. RNAβ
5 gives rise to two subgenomic ("sg") RNA-β mRNA: sgRNAβ1 and sgRNAβ2, which have transcription initiation sites at nucleotides 789 and 2327, respectively, of RNA-β. sgRNAβ1 directs translation of the βb protein, while sgRNAβ2 translates the βc and βd proteins and a 23 kDa protein (designated βd') which is a readthrough product of the amber stop codon of the βd ORF (Zhou *et al.*, 1996). In the presence of both
10 RNAα and RNAγ, RNAβ containing gene replacements of either βa or βb with the firefly luciferase (*luc*) gene mediate expression of the *luc* gene upon transfection into tobacco leaf mesophyll and maize protoplasts (Joshi *et al.*, 1998). In-frame insertions of the chloramphenicol acetyl transferase ("CAT") reporter gene in the βb, βc, βd, and βd' ORFs of RNA-β, when coinoculated into barley cultivar protoplasts with RNA-α
15 and RNA-γ, resulted in the expression of active CAT from insertions in βb, βc, βd, and βd' (Zhou *et al.*, 1996).

The coat protein may be encoded on a polynucleotide that is distinct from the polynucleotide encoding the protein of interest. The coat protein may be encoded on
20 a subgenomic RNA that is distinct from the subgenomic RNA encoding the protein of interest. In a preferred embodiment the viral genome is the genome of a hordeivirus, and the coat protein is encoded on RNAβ that is distinct from RNAα or RNAγ encoding the protein of interest.

The non-expression of the coat protein is the total absence of the coat protein
25 (e.g. caused by the deletion of the coat protein gene) or a decrease of the expression of the coat protein, as compared to a wild-type virus, or the expression of a mutant form of coat protein, or a combination of any of the above phenomenon. The non-expression of the coat protein may be brought about by deleting the coat protein gene, or by deleting a fragment of the coat protein gene whereby sufficient sequence
30 essential to cause to increase expression from the promoter. The sequences important for causing the increased expression of the protein of interest can be easily determined by one of ordinary skill in the art by creating a series of nested deletions of the coat

protein gene and testing to determine whether the expression of the protein of interest is increased.

The basis by which the deletion or mutation of the coat protein, or fragment thereof, causes an increased expression of the protein of interest may be due to the

5   competition of the coat protein with the polynucleotide encoding the protein of interest for one or more factor or for access to the translation machinery. Another basis may be that the regulation of the promoter operatively linked to the transcription unit may be regulated or modulated by the presence or absence of the coat protein either directly or indirectly. Another further basis may be that certain nucleic acid

10 region(s) of the coat protein regulates or modulates the expression of the promoter operatively linked to the transcription unit either directly or indirectly. That the non-expression of the coat protein causes an increased expression of the transcription unit may be due to any combination of the reasons cited above.

The duplicated genomic nucleic component may be a subgenomic RNA. The

15 duplicated genomic nucleic component may be a duplicated subgenomic RNA of a multipartite virus. The promoters of all the duplicated genomic nucleic acid components may be identical or different to each other. The ORFs of all the duplicated genomic nucleic acid components can be identical or different to each other.

20 There can be one or more duplicated genomic nucleic acid components. The viral genome may further comprise additional duplicated genomic nucleic acid component(s), wherein each of the additional duplicated genomic nucleic acid component encodes an transcriptional unit operatively linked to a promoter, wherein each transcriptional unit may be non-identical to the transcriptional unit (s) of the

25 other duplicated genomic nucleic acid component(s), wherein each promoter may be non-identical to the promoter(s) of the other duplicated genomic nucleic acid component(s). If the viral genome is the genome of a hordeivirus, such as a BSMV, the duplicated genomic nucleic acid component is preferably duplicated RNAγ. If the viral genome is the genome of a BSMV, the promoter is preferably the promoter of

30 the γb gene.

If the duplicated genomic nucleic acid and component is a duplicated subgenomic RNA of a hordeivirus, such as a BSMV, the viral genome plus any

duplicated subgenomic RNA can be packaged into a recombinant virus, or recombinant viral particle using the Argentina Mild strain of BSMV.

The present invention is also directed to compositions and methods for decreasing the expression of a plant gene (or a gene of interest to be silenced or a

5   target gene) in order to bring about a desired phenotypic or biochemical change of a plant host. The present invention is also directed to a method of changing the phenotype or biochemistry of a plant host, a method of determining a change in phenotype or biochemistry in a plant host, a method of determining the presence of a trait in a plant host, and a method of determining the function of a nucleic acid

10   sequence. The method comprise the steps of expressing permanently or transiently a nucleic acid sequence in a sense or antisense orientation in a plant host, identifying changes in the plant host and correlating the expression and the changes.

The present invention provides a method of infecting a plant host by a recombinant plant viral nucleic acid with contains one or more non-native nucleic acid

15   sequences, or by a recombinant plant virus containing a recombinant plant nucleic acid. The non-native nucleic acids are subsequently transcribed or expressed in the infected host plant. The products of the non-native nucleic acid sequences result in changing phenotypic traits in the plant host, affecting biochemical pathways within the plant, or affecting endogenous expression within the plant.

20   The invention provides for recombinant plant viruses comprising a recombinant viral vector capable of decreasing the expression of a plant gene. The decreasing of the expression of a plant gene may take place in a host. The recombinant plant viruses or recombinant viral vectors may infect a single host cell or systemically infect a plant. Thus by employing the recombinant plant viruses or

25   recombinant viral vectors of the invention, by decreasing or totally shutting down the expression of one or more plant genes the phenotype or one or more characteristic of a plant may be altered. The present invention also provides for methods involving the use of such recombinant plant viruses and recombinant viral vector which bring about the decreased expression of a plant gene or the altering of the phenotype or one or

30   more characteristic of a plant.

The stop codon, also known as a terminating or termination codon, may be TAA, TAG, or TGA, as read in DNA from 5' to 3', or UAA, UAG, or UGA, as read in RNA from 5' to 3'.

The selection of the genetic backbone for the viral vectors of the instant invention may depend on the plant host used. The plant host may be a monocotyledonous or dicotyledonous plant, plant tissue, or plant cell. Typically, plants of commercial interest, such as food crops, seed crops, oil crops, ornamental crops and forestry crops are preferred. For example, wheat, rice, corn, potato, barley, tobacco, soybean canola, maize, oilseed rape, lilies, grasses, orchids, irises, onions, palms, tomato, the legumes, or *Arabidopsis*, can be used as a plant host. Host plants may also include those readily infected by an infectious virus, such as *Nicotiana*, preferably, *Nicotiana benthamiana*, or *Nicotiana clevelandii*.

One feature of the present invention is the use of plant viral nucleic acids which comprise one or more non-native nucleic acid sequences capable of being transcribed in a plant host. These nucleic acid sequences may be native nucleic acid sequences that occur in a host plant. Preferably, these nucleic acid sequences are non-native nucleic acid sequences that do not normally occur in a host plant. For example, the plant viral vectors may contain sequences from more than one virus, including viruses from more than one taxonomic group. The plant viral nucleic acids may also contain sequences from non-viral sources, such as foreign genes, regulatory sequences, fragments thereof from bacteria, fungi, plants, animals or other sources. These foreign sequences may encode commercially useful proteins, polypeptides, or fusion products thereof, such as enzymes, antibodies, hormones, pharmaceuticals, vaccines, pigments, antimicrobial polypeptides, and the like. Or they may be sequences that regulate the transcription or translation of viral nucleic acids, package viral nucleic acid, and facilitate systemic infection in the host, among others.

In some embodiments of the instant invention, the plant viral vectors may comprise one or more additional native or non-native subgenomic promoters which are capable of transcribing or expressing adjacent nucleic acid sequences in the plant host. These non-native subgenomic promoters are inserted into the plant viral nucleic acids without destroying the biological function of the plant viral nucleic acids using known methods in the art. For example, the CaMV promoter can be used when plant cells are to be transfected. The subgenomic promoters are capable of functioning in the specific host plant. For example, if the host is tobacco, TMV, tomato mosaic virus, or other viruses containing at least one subgenomic promoter may be utilized. The inserted subgenomic promoters should be compatible with the TMV nucleic acid

and capable of directing transcription or expression of adjacent nucleic acid sequences in tobacco. It is specifically contemplated that two or more heterologous non-native subgenomic promoters may be used. The non-native nucleic acid sequences may be transcribed or expressed in the host plant under the control of the subgenomic

5   promoter to produce the products of the nucleic acids of interest.

In some embodiments of the instant invention, the recombinant plant viral nucleic acids may be further modified by conventional techniques to delete all or part of the native coat protein coding sequence or put the native coat protein coding sequence under the control of a non-native plant viral subgenomic promoter. If it is

10   deleted or otherwise inactivated, a non-native coat protein coding sequence is inserted under control of one of the non-native subgenomic promoters, or optionally under control of the native coat protein gene subgenomic promoter. Thus, the recombinant plant viral nucleic acid contains a coat protein coding sequence, which may be native or a nonnative coat protein coding sequence, under control of one of the native or non-

15   native subgenomic promoters. The native or non-native coat protein gene may be utilized in the recombinant plant viral nucleic acid. The non-native coat protein, as is the case for the native coat protein, may be capable of encapsidating the recombinant plant viral nucleic acid and providing for systemic spread of the recombinant plant viral nucleic acid in the host plant.

20   In some embodiments of the instant invention, nucleic sequences encoding reporter protein(s) or antibiotic/herbicide resistance gene(s) may be constructed as carrier protein(s) for the polypeptides of interest, which may facilitate the detection of polypeptides of interest. For example, green fluorescent protein (GFP) may be simultaneously expressed with polypeptides of interest. In another example, a

25   reporter gene, β-glucuronidase (GUS) may be utilized. In another example, a drug resistance marker, such as a gene whose expression results in kanamycin resistance, may be used.

Since the RNA genome is typically the infective agent, the transcriptional unit is positioned adjacent a suitable promoter so that the RNA is produced in the

30   production cell. The RNA is capped using conventional techniques, if the capped RNA is the infective agent. In addition, the capped RNA can be packaged *in vitro* with added coat protein from TMV to make assembled virions. These assembled virions can then be used to inoculate plants or plant tissues. Alternatively, an

uncapped RNA may also be employed in the embodiments of the present invention. Contrary to the practiced art in scientific literature and in issued patent (Ahlquist *et al.*, U.S. Patent No. 5,466,788), uncapped transcripts for virus expression vectors are infective on both plants and in plant cells. Capping is not a prerequisite for

5   establishing an infection of a virus expression vector in plants, although capping increases the efficiency of infection. In addition, nucleotides may be added between the transcription start site of the promoter and the start of the cDNA of a viral nucleic acid to construct an infectious viral vector. One or more nucleotides may be added. In some embodiments of the present invention, the inserted nucleotide sequence may

10   contain a G at the 5'-end. Alternatively, the inserted nucleotide sequence may be GNN, GTN, or their multiples, $(GNN)_x$ or $(GTN)_x$.

The polynucleotides of the subject invention may be encoded in RNA or DNA or any synthetic nucleic acid, single- or double-stranded, linear or circular, capable of expression into RNA in a eukaryotic host, such as a yeast, such as *Sacchromyces*

15   *cerevisiae*, or a prokaryotic host, such as a bacteria, as *Escherichia coli*. Depending on the desired host to be used the necessary nucleotide structures necessary for maintenance in the host, such as origin of replication sites, amplifiable selectable markers, etc., and expression in the host, such as promoters, activation sites, etc. need to be present on the RNA or DNA. Such are known to one of ordinary of the art (see

20   Old and Primrose, *Principles of Gene Manipulation* 5th ed., Blackwell Science, Oxford, U.K. (1994)).

In some embodiments of the instant invention, more than one nucleic acid is prepared for a multipartite viral vector construct. In this case, each nucleic acid may require its own origin of assembly. Each nucleic acid could be prepared to contain a

25   subgenomic promoter and a non-native nucleic acid. Alternatively, the insertion of a non-native nucleic acid into the nucleic acid of a monopartite virus may result in the creation of two nucleic acids (i.e., the nucleic acid necessary for the creation of a bipartite viral vector). This would be advantageous when it is desirable to keep the replication and transcription or expression of the nucleic acid of interest separate from

30   the replication and translation of some of the coding sequences of the native nucleic acid.

The host can be any cell. The host can be any animal or plant cell. The plant cell may a protoplast, a recombinant cell, a transgenic cell, a non-transgenic cell, or a

cell that is part of a cell culture, cell tissue, plant organ, or an entire plant organism. A protoplast is a plant cell that has the cell wall removed. The plant cell can be a dicot or a monocot plant cell. The host may be of a species or strain that can be infected with a viral genome or a recombinant virus obtained from a virus that can infect the

5   host.

Plant hosts include plants of commercial interest, such as food crops, seed crops, oil crops, ornamental crops and forestry crops. For example, wheat, rice, corn, potatoes, barley, tobaccos, soybean canola, maize, oilseed rape, *Arabidopsis*, *Nicotiana* can be selected as a host plant. In particular, host plants capable of being

10   infected by a virus containing a recombinant viral nucleic acid are preferred. Preferred host plants include *Nicotiana*, preferably, *Nicotiana benthamiana*, or *Nicotiana cleavlandii*.

Individual clones may be transfect into the plant host: 1) protoplasts; 2) whole plants; or 3) plant tissues, such as leaves of plants (Dijkstra *et al.*, *Practical Plant*

15   *Virology: Protocols and Exercises*, Springer Verlag (1998); *Plant Virology Protocol: From Virus Isolation to Transgenic Resistance* in *Methods in Molecular Biology*, Vol. 81, Foster and Taylor, Ed., Humana Press (1998)). In some embodiments of the instant invention, the delivery of the recombinant plant nucleic acid into the plant may be affected by the inoculation of *in vitro* transcribed RNA, inoculation of virions, or

20   internal inoculation of plant cells from nuclear cDNA, or the systemic infection resulting from any of these procedures. In all cases, the co-infection may lead to a rapid and pervasive systemic expression of the desired nucleic acid sequences in plant cells.

The host can be infected with a recombinant viral nucleic acid or a

25   recombinant plant virus by conventional techniques. Suitable techniques include, but are not limited to, leaf abrasion, abrasion in solution, high velocity water spray, and other injury of a host as well as imbibing host seeds with water containing the recombinant viral RNA or recombinant plant virus. More specifically, suitable techniques include:

30   (a)   Hand Inoculations. Hand inoculations are performed using a neutral pH, low molarity phosphate buffer, with the addition of celite or carborundum (usually about 1%). One to four drops of the preparation is put onto the upper surface of a leaf and gently rubbed.

(b) Mechanized Inoculations of Plant Beds. Plant bed inoculations are performed by spraying (gas-propelled) the vector solution into a tractor-driven mower while cutting the leaves. Alternatively, the plant bed is mowed and the vector solution sprayed immediately onto the cut leaves.

5   (c) High Pressure Spray of Single Leaves. Single plant inoculations can also be performed by spraying the leaves with a narrow, directed spray (50 psi, 6-12 inches from the leaf) containing approximately 1% carborundum in the buffered vector solution.

(d) Vacuum Infiltration. Inoculations may be accomplished by subjecting a host
10   organism to a substantially vacuum pressure environment in order to facilitate infection.

(e) High Speed Robotics Inoculation. Especially applicable when the organism is a plant, individual organisms may be grown in mass array such as in microtiter plates. Machinery such as robotics may then be used to transfer the nucleic
15   acid of interest.

(f) Ballistics (High Pressure Gun) Inoculation. Single plant inoculations can also be performed by particle bombardment. A ballistics particle delivery system (BioRad Laboratories, Hercules, (A) can be used to transfect plants such as *N. benthamiana* as described previously (Nagar *et al.*, *Plant Cell*, 7:705-719
20   (1995)).

An alternative method for introducing recombinant viral nucleic acids into a plant host is a technique known as agroinfection or *Agrobacterium*-mediated transformation (also known as Agro-infection) as described by Grimsley, *et al.*, *Nature* 325:177 (1987). This technique makes use of a common feature of
25   *Agrobacterium* which colonizes plants by transferring a portion of their DNA (the T-DNA) into a host cell, where it becomes integrated into nuclear DNA. The T-DNA is defined by border sequences which are 25 base pairs long, and any DNA between these border sequences is transferred to the plant cells as well. The insertion of a recombinant plant viral nucleic acid between the T-DNA border sequences results in
30   transfer of the recombinant plant viral nucleic acid to the plant cells, where the recombinant plant viral nucleic acid is replicated, and then spreads systemically through the plant. Agro-infection has been accomplished with potato spindle tuber viroid (PSTV) (Gardner *et al.*, *Plant Mol. Biol.* 6:221 (1986); CaV (Grimsley *et al.*,

Proc. Natl. Acad. Sci. USA 83:3282 (1986)); MSV (Grimsley *et al., Nature* 325:177 (1987)), and Lazarowitz, S., *Nucl. Acids Res.* 16:229 (1988)) digitaria streak virus (Donson *et al., Virology* 162:248 (1988)), wheat dwarf virus (Hayes *et al., J. Gen. Virol.* 69:891 (1988)) and tomato golden mosaic virus (TGMV) (Elmer *et al., Plant*

5 *Mol. Biol.* 10:225 (1988) and Gardiner *et al., EMBO J.* 7:899 (1988)). Therefore, agro-infection of a susceptible plant could be accomplished with a virion containing a recombinant plant viral nucleic acid based on the nucleotide sequence of any of the above viruses. Particle bombardment or electrosporation or any other methods known in the art may also be used.

10 In some embodiments of the instant invention, infection may also be attained by placing a selected nucleic acid sequence into an organism such as *E. coli,* or yeast, either integrated into the genome of such organism or not, and then applying the organism to the surface of the host organism. Such a mechanism may thereby produce secondary transfer of the selected nucleic acid sequence into a host organism. This is

15 a particularly practical embodiment when the host organism is a plant. Likewise, infection may be attained by first packaging a selected nucleic acid sequence in a pseudovirus. Such a method is described in WO 94/10329. Though the teachings of this reference may be specific for bacteria, those of skill in the art will readily appreciate that the same procedures could easily be adapted to other organisms.

20 Plant may be grown from seed in a mixture of "Peat-Lite Mix™ (Speedling, Inc. Sun City, Fl) and Nutricote™ controlled release fertilizer 14-14-14 (Chiss-Asahi Fertilizer Co., Tokyo, Japan). Plants may be grown in a controlled environment provided 16 hours of light and 8 hours of darkness. Sylvania "Gro-Lux/Aquarium" wide spectrum 40 watt fluorescent grow lights. (Osram Sylvania Products, Inc.

25 Danvers, MA) may be used. Temperatures may be kept at around 80° F during light hours and 70° F during dark hours. Humidity may be between 60 and 85%.

A library of sequence inserts from one or more donor organism(s) may be constructed using any of the following methods.

30 A recombinant plant viral nucleic acid may be prepared by cloning a viral nucleic acid. If the viral nucleic acid is DNA, it can be cloned directly into a suitable vector using conventional techniques. One technique is to attach an origin of replication to the viral DNA which is compatible with the cell to be transfected. In

this manner, DNA copies of the chimeric nucleotide sequence are produced in the transfected cell. If the viral nucleic acid is RNA, a DNA copy of the viral nucleic acid is first prepared by well-known procedures. For example, the viral RNA is transcribed into DNA using reverse transcriptase to produce subgenomic DNA pieces,

5   and a double-stranded DNA may be produced using DNA polymerases. The cDNA is then cloned into appropriate vectors and cloned into a cell to be transfected. In some instances, cDNA is first attached to a promoter which is compatible with the production cell. The recombinant plant viral nucleic acid can then be cloned into any suitable vector which is compatible with the production cell. Alternatively, the

10   recombinant plant viral nucleic acid is inserted in a vector adjacent a promoter which is compatible with the production cell. In some embodiments, the cDNA ligated vector may be directly transcribed into infectious RNA *in vitro* and inoculated onto the plant host. The cDNA pieces are mapped and combined in proper sequence to produce a full-length DNA copy of the viral RNA genome, if necessary.

15   The donor organism from which a library of sequence inserts is derived includes Kingdom *Monera,* Kingdom *Protista,* Kingdom *Fungi,* Kingdom *Plantae* and Kingdom *Animalia.* Kingdom *Monera* includes subkingdom *Archaebacteriobionta* (archaebacteria): division *Archaebacteriophyta* (methane, salt and sulfolobus bacteria); subkingdom *Eubacteriobionta* (true bacteria): division

20   *Eubacteriophyta*; subkingdom *Viroids*; and subkingdom *Viruses.* Kingdom *Protista* includes subkingdom *Phycobionta:* division *Xanthophyta* 275 (yellow-green algae), division *Chrysophyta* 400 (golden-brown algae), division *Dinophyta* (Pyrrhophyta) 1,000 (dinoflagellates), division *Bacillariophyta* 5,500 (diatoms), division *Cryptophyta* 74 (cryptophytes), division *Haptophyta* 250 (haptonema organisms),

25   division *Euglenophyta* 550 (euglenoids), division *Chlorophyta,* class *Chlorophyceae* 10,000 (green algae), class *Charophyceae* 200 (stoneworts), division *Phaeophyta* 900 (brown algae), and division *Rhodophyta* 2,500 (red algae); subkingdom *Mastigobionta* 960: division *Chytridiomycota* 750 (chytrids), and division *Oomycota* (water molds) 475; subkingdom *Myxobionta* 320: division *Acrasiomycota* (cellular

30   slime molds) 21, and division *Myxomycota* 500 (true slime molds). Kingdom *Fungi* includes division *Zygomycota* 570 (coenocytic fungi): subdivision *Zygomycotina*; and division *Eumycota* 350 (septate fungi): subdivision *Ascomycotina* 56,000 (cup fungi), subdivision *Basidiomycotina* 25,000 (club fungi), subdivision *Deuteromycotina*

22,000 (imperfect fungi), and subdivision *Lichenes* 13,500. Kingdom Plantae includes division *Bryophyta, Hepatophyta, Anthocerophyta, Psilophyta, Lycophyta, Sphenophyta, Pterophyta, Coniferophyta, Cycadeophyta, Ginkgophyta, Gnetophyta* and *Anthophyta*. Kingdom *Animalia* includes: *Porifera* (Sponges), *Cnidaria*

5   (Jellyfishes),. *Ctenophora* (Comb Jellies), *Platyhelminthes* (Flatworms), *Nemertea* (Proboscis Worms), *Rotifera* (Rotifers), *Nematoda* (Roundworms), *Mollusca* (Snails, Clams, Squid & Octopus), *Onychophora* (Velvet Worms), *Annelida* (Segmented Worms), *Arthropoda* (Spiders & Insects), *Phoronida, Bryozoa* (Bryozoans), *Brachiopoda* (Lamp Shells), *Echinodermata* (Sea Urchins & starfish), and *Chordata*

10 (Vertebrata-Fish, Birds, Reptiles, Mammals). A preferred donor organism is human. Host organisms are those capable of being infected by an infectious RNA or a virus containing a recombinant viral nucleic acid. Host organisms include organisms from *Monera, Protista, Fungi* and *Animalia*. Preferred host organisms are organisms from *Fungi*, such as yeast (for example, *S. cerevisiae*) and *Anamalia*, such as insects (for

15 example, *C. elegans*).

To prepare a DNA insert comprising a nucleic acid sequence of a donor organism, the first step is to construct a cDNA library, a genomic DNA library, or a pool of mRNA of the donor organism. Full-length cDNAs or genomic DNA can be obtained from public or private repositories. For example, cDNA and genomic

20 libraries from bovine, chicken, dog, drosophila, fish, frog, human, mouse, porcine, rabbit, rat, and yeast; and retroviral libraries can be obtained from Clontech (Palo Alto, CA). Alternatively, cDNA library can be prepared from a field sample by methods known to a person of ordinary skill, for example, isolating mRNAs and transcribing mRNAs into cDNAs by reverse transcriptase (*see, e.g.,* Sambrook *et al.,*

25 *Molecular Cloning: A Laboratory Manual* (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989), or *Current Protocols in Molecular Biology,* F. Ausubel *et al.,* ed. Greene Publishing and Wiley-Interscience, New York (1987)). Genomic DNAs represented in BAC (bacterial artificial chromosome), YAC (yeast artificial chromosome), or TAC (transformation-competent artificial chromosome, Lin *et al.,*

30 *Proc. Natl. Acad. Sci. USA,* 96:6535-6540 (1999)) libraries can be obtained from public or private repositories.

Alternatively, a pool of genes, which are overexpressed in a tumor cell line compared with a normal cell line, can be prepared or obtained from public or private

repositories. Zhang *et al.* (*Science,* 276: 1268-1272 (1997)) report that using a method of serial analysis of gene expression (SAGE) (Velculescu *et al, Cell,* 88:243 (1997)), 500 transcripts that were expressed at significantly different levels in normal and neoplastic cells were identified. The expression of DNAs that overexpresses in a

5   tumor cell line in a host organism may cause changes in the host organism, thus a pool of such DNAs is another source for DNA inserts for this invention. The BAC/YAC/TAC DNAs, DNAs or cDNAs can be mechanically size-fractionated or digested by an enzyme to smaller fragments. The fragments are ligated to adapters with cohesive ends, and shotgun-cloned into recombinant viral nucleic acid vectors.

10   Alternatively, the fragments can be blunt-end ligated into recombinant viral nucleic acid vectors. Recombinant viral nucleic acids containing a nucleic acid sequence derived from the cDNA library or genomic DNA library is then constructed using conventional techniques. The recombinant viral nucleic acid vectors produced comprise the nucleic acid insert derived from the donor organism. The nucleic acid

15   sequence of the recombinant viral nucleic acid is transcribed as RNA in a host organism; the RNA is capable of regulating the expression of a phenotypic trait by a positive or anti sense mechanism. The nucleic acid sequence may also regulate the expression of more than one phenotypic trait. Nucleic acid sequences from *Monera, Protista, Fungi, Plantae* and *Animalia* may be used to assemble the DNA libraries.

20   This method may thus be used to discover useful dominant gene phenotypes from DNA libraries through the gene expression in a host organism.

In the case of using plant as a donor organism, the donor plant and the host plant may be genetically remote or unrelated: they may belong to different genus, family, order, class, subdivision, or division. Donor plants include plants of

25   commercial interest, such as food crops, seed crops, oil crops, ornamental crops and forestry crops. For example, wheat, rice, corn, potatoes, barley, tobaccos, soybean canola, maize, oilseed rape, *Arabidopsis, Nicotiana* can be selected as a donor plant.

To prepare a DNA insert comprising a nucleic acid sequence of a donor plant, the first step is typically to construct a library of cDNAs, genomic DNAs, or a pool of

30   RNAs of the plant of interest. Full-length cDNAs can be obtained from public or private repositories, for example, cDNA library of *Arabidopsis thaliana* can be obtained from the *Arabidopsis* Biological Resource Center. Alternatively, cDNA library can be prepared from a field sample by methods known to a person of ordinary

skill, for example, isolating mRNAs and transcribing mRNAs into cDNAs by reverse transcriptase (*see, e.g.,* Sambrook *et al., Molecular Cloning: A Laboratory Manual* (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989), or *Current Protocols in Molecular Biology,* F. Ausubel *et al.,* ed. Greene Publishing and Wiley-Interscience,

5   New York (1987)). Genomic DNAs represented in BAC (bacterial artificial chromosome), YAC (yeast artificial chromosome), or TAC (transformation-competent artificial chromosome, Liu *et al., Proc. Natl. Acad. Sci. USA,* 96:6535-6540 (1999)) libraries can be obtained from public or private repositories, for example, the *Arabidopsis* Biological Resource Center. The BAC/YAC/TAC DNAs

10   or cDNAs can be mechanically size-fractionated or digested by an enzyme to smaller fragments. The fragments are ligated to adapters with cohesive ends, and shotgun-cloned into recombinant viral nucleic acid vectors. Alternatively, the fragments can be blunt-end ligated into recombinant viral nucleic acid vectors. Recombinant plant viral nucleic acids containing a nucleic acid sequence derived from the cDNA library

15   or genomic DNA library is then constructed using conventional techniques. The recombinant viral nucleic acid vectors produced comprise the nucleic acid insert derived from the donor plant. The nucleic acid sequence of the recombinant viral nucleic acid is transcribed as RNA in a host plant; the RNA is capable of regulating the expression of a phenotypic trait by a positive or anti sense mechanism. The

20   nucleic acid sequence may also code for the expression of more than one phenotypic trait. Sequences from wheat, rice, corn, potato, barley, tobacco, soybean, canola, maize, oilseed rape, *Arabidopsis,* and other crop species may be used to assemble the DNA libraries. This method may thus be used to search for useful dominant gene phenotypes from DNA libraries through the gene expression.

25   Those skilled in the art will understand that these embodiments are representative only of many constructs suitable for the instant invention. All such constructs are contemplated and intended to be within the scope of the present invention. The invention is not intended to be limited to any particular viral constructs but specifically contemplates using all operable constructs. A person

30   skilled in the art will be able to construct the plant viral nucleic acids based on molecular biology techniques well known in the art. Suitable techniques have been described in Sambrook *et al.* (2nd ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor (1989); *Methods in Enzymol.* (Vols. 68, 100, 101, 118, and 152-155) (1979,

1983, 1986 and 1987); and *DNA Cloning*, D.M. Clover (ed.), IRL Press, Oxford

(1985); Walkey, *Applied Plant Virol.*, Chapman & Hall (1991); Matthews, *Plant

Virol.*, 3rd Ed., Academic Press, San Diego (1991); Turpen *et al.*, *J. of Virological

Methods*, 42:227-240 (1993); U.S. Patent Nos. 4,885,248, 5,173,410, 5,316,931,

5   5,466,788, 5,491,076, 5,500,360, 5,589,367, 5,602,242, 5,627,060, 5,811,653,

5,866,785, 5,889,190, 5,589,367, and 5,977,438. Nucleic acid manipulations and

enzyme treatments are carried out in accordance with manufacturers' recommended

procedures in making such constructs.

.   Any of the following methods for detecting phenotypic or biochemical

10   changes as a result of expression of the transcriptional unit may be used.

After a plant host is infected with individual clone of the library, one or more

phenotypic or biochemical changes may be detected. The phenotypic changes in a

plant host may be determined by any known methods in the art. Phenotypic changes

may include growth rate, color, or morphology changes. Typically, these methods

15   include visual, macroscopic or microscopic analysis. For example, growth changes,

such as stunting, color changes (e.g. leaf yellowing, mottling, bleaching, chlorosis)

among others are easily visualized. Examples of morphological changes include,

developmental defects, wilting, necrosis, among others.

Biochemical changes can be determined by any analytical methods known in

20   the art for detecting, quantitating, or isolating DNA, RNA, proteins, antibodies,

carbohydrates, lipids, and small molecules. Selected methods may include Northern,

Western blotting, matrix-assisted laser desorption time of flight mass spectrometry

(MALDI-TOF), LC/MS, GC/MS, two-dimensional IEF/SDS-PAGE, enzyme -linked

immunosorbent assay (ELISA), etc. In particular, suitable methods may be performed

25   in a high-throughput, fully automated fashion using robotics. Examples of

biochemical changes may include the accumulation of substrates or products from

enzymatic reactions, changes in biochemical pathways, inhibition or augmentation of

endogenous gene expression in the cytoplasm of cells, changes in the RNA or protein

profile.   For example, the clones in the viral vector library may be functionally

30   classified based on metabolic pathway affected or visual/selectable phenotype

produced in the organism. This process enables a rapid determination of gene

function for unknown nucleic acid sequences of a donor organism as well as a host

organism. Furthermore, this process can be used to rapidly confirm function of full-

length DNA's of unknown function. Functional identification of unknown nucleic acid sequences in a library of one organism may then rapidly lead to identification of similar unknown sequences in expression libraries for other organisms based on sequence homology. Such information is useful in many aspects including in human

5   medicine.

The biochemical or phenotypic changes in the infected host plant may be correlated to the biochemistry or phenotype of a host plant that is uninfected. Optionally, the biochemical or phenotypic changes in the infected host plant is further correlated to a host plant that is infected with a viral vector that contains a control

10   nucleic acid of a known sequence. The control nucleic acid may have similar size but is different in sequence from the nucleic acid insert derived from the library. For example, if the nucleic acid insert derived from the library is identified as encoding a GTP binding protein in an antisense orientation, a nucleic acid derived from a gene encoding green fluorescent protein can be used as a control nucleic acid. Green

15   fluorescent protein is known not to have the same effect as the GTP binding protein when expressed in a host plant.

In some embodiments, the phenotypic or biochemical trait may be determined by complementation analysis, that is, by observing the endogenous gene or genes whose function is replaced or augmented by introducing the nucleic acid of interest.

20   A discussion of such phenomenon is provided by Napoli *et al., The Plant Cell* 2:279-289 (1990). The phenotypic or biochemical trait may also be determined by (1) analyzing the biochemical alterations in the accumulation of substrates or products from enzymatic reactions according to any means known by those skilled in the art; (2) by observing any changes in biochemical pathways which may be modified in a

25   host organism as a result of expression of the nucleic acid; (3) by utilizing techniques known by those skilled in the art to observe inhibition of endogenous gene expression in the cytoplasm of cells as a result of expression of the nucleic acid.; (4) by utilizing techniques known by those skilled in the art to observe changes in the RNA or protein profile as a result of expression of the nucleic acid; or (5) by selection of organisms

30   capable of growing or maintaining viability in the presence of noxious or toxic substances, such as, for example, pharmaceutical ingredients.

One useful means to determine the function of nucleic acids transfected into a host plant is to observe the effects of gene silencing. Traditionally, functional gene

knockout has been achieved following inactivation due to insertion of transposable elements or random integration of T-DNA into the chromosome, followed by characterization of conditional, homozygous-recessive mutants obtained upon backcrossing. Some teachings in these regards are provided by WO97/42210 which is

5 herein incorporated by reference. As an alternative to traditional knockout analysis, an EST/DNA library from a donor organism, may be assembled into a viral transcription plasmid. The nucleic acid sequences in the transcription plasmid library may then be introduced into host cells as part of a functional RNA virus which post-transcriptionally silences the homologous target gene. The EST/DNA sequences may

10 be introduced into a viral vector in either the plus or anti sense orientation, and the orientation can be either directed or random based on the cloning strategy. A high-throughput, automated cloning scheme based on robotics may be used to assemble and characterize the library. Alternatively, the EST/cDNA sequences can be inserted into the genomic RNA of a viral vector such that they are represented as genomic

15 RNA during the viral replication in host cells. The library of EST clones is then transcribed into infectious RNAs and inoculated onto a host organism susceptible to viral infection. The viral RNAs containing the EST/cDNA sequences contributed from the original library are now present in a sufficiently high concentration in the cytoplasm of host organism cells such that they cause post-transcriptional gene

20 silencing of the endogenous gene in a host organism. Since the replication mechanism of the virus produces both sense and antisense RNA sequences, the orientation of the EST/cDNA insert is normally irrelevant in terms of producing the desired phenotype in the host organism.

The present invention provides a method to express transiently viral-derived

25 positive sense or antisense RNAs in transfected plants. Such method is much faster than the time required to obtain genetically engineered antisense transgenic organisms. Systemic infection and expression of viral antisense RNA occurs as short as several days post inoculation, whereas it takes several months or longer to create a single transgenic organism. The invention provides a method to identify genes

30 involved in the regulation of growth by inhibiting the expression of specific endogenous genes using viral vectors. This invention provides a method to characterize specific genes and biochemical pathways in donor organisms or in host plants using an RNA viral vector.

It is known that silencing of endogenous genes can be achieved with homologous sequences from the same plant family. For example, Kumagai *et al.*, *(Proc. Natl. Acad. Sci. USA* 92:1679 (1995)) report that the *Nicotiana benthamiana* gene for phytoene desaturase (PDS) was silenced by transfection with a viral RNA

5   derived from a clone containing a partial tomato *(Lycopersicon esculentum)* cDNA encoding PDS being in an antisense orientation. Kumagai *et al.* demonstrate that gene encoding PDS from one plant can be silenced by transfecting a host plant with a nucleic acid of a known sequence, namely, a PDS gene, from a donor plant of the same family. The present invention provides a method of silencing a gene in a host

10   organism by transfecting a non-plant host organism with a viral nucleic acid comprising a nucleic acid insert derived from a cDNA library or a genomic DNA library or a pool of RNA from a non-plant organism. Different from Kumagai *et al*, the sequence of the nucleic acid insert in the present invention does not need to be identified prior to the transfection. Another feature of the present invention is that it

15   provides a method to silence a conserved gene of a non-plant kingdom; the antisense transcript of an organism results in reducing expression of the endogenous gene of a host organism from *Monera*, Protista, *Fungi* and *Animalia*. The invention is exemplified by GTP binding proteins. In eukaryotic cells, GTP-binding proteins function in a variety of cellular processes, including signal transduction, cytoskeletal

20   organization, and protein transport. Low molecular weight (20-25 kDa) of GTP-binding proteins include *ras* and its close relatives (for example, *Ran*), *rho* and its close relatives, the *rab* family, and the ADP-ribosylation factor (ARF) family. The heterotrimeric and monomeric GTP-binding proteins that may be involved in secretion and intracellular transport are divided into two structural classes: the *rab* and

25   the ARF families. The ARFs from many organisms have been isolated and characterized. The ARFs share structural features with both the *ras* and trimeric GTP-binding protein families. The present invention demonstrates that the gene of a plant, such as barley, can be silenced by transfection with infectious RNAs from a clones containing a full-length or partial cDNA of the PDS open reading frame in a sense

30   orientation, derived from a plant of a different family, such as rice and maize. The present invention also demonstrates that PDS proteins are highly homologous in plants, not only at the amino acid level, but also at the nucleic acid level. The present invention thus provides a method to silence a conserved gene in a host organism, by

transfecting the host with infectious RNAs derived from a homologous gene of a different plant organism.

A library of human nucleic acid sequences is cloned into vectors. The vectors are applied to the host to obtain infection. Each infected host is grown with an
5   uninfected host and a host infected with a null vector. A null vector will show no phenotypic or biochemical change other than the effects of the virus itself. Each host is observed daily or regularly for visual differences between the infected host and its two controls. In each host displaying an observable phenotypic or biochemical change a trait is identified. The donor nucleic acid sequence is identified, the full-
10   length gene sequence is obtained and the full-length gene in the host is obtained, if a gene from the host is associated with the trait. Both genes are sequenced and homology is determined. A variety of biochemical tests may also be made on the host or host tissue depending on the information that is desired. A variety of phenotypic changes or traits and biochemical tests are set forth in this document. A functional
15   gene profile can be obtained by repeating the process several times.

Large amounts of DNA sequence information are being generated in the public domain, which may be entered into a relational database. Links may be made between sequences from various species predicted to carry out similar biochemical or regulatory functions. Links may also be generated between predicted enzymatic
20   activities and visually displayed biochemical and regulatory pathways. Likewise, links may be generated between predicted enzymatic or regulatory activity and known small molecule inhibitors, activators, substrates or substrate analogs. Phenotypic data from expression libraries expressed in transfected hosts may be automatically linked within such a relational database. Genes with similar predicted roles of interest in
25   other organisms may be rapidly discovered.

The present invention is also directed to a method of changing the phenotype or biochemistry of a plant by expressing transiently a nucleic acid sequence from a donor plant in a sense or antisense orientation in a host plant, which inhibits an endogenous gene expression in the meristem of the host plant. The one or more
30   phenotypic or biochemical changes in the host plant are detected by methods as describes previously. Transient expressing a nucleic acid sequence in a host plant can affect the gene expression in meristem. Meristems are of interest in plant development because plant growth is driven by the formation and activity of

meristems throughout the entire life cycle. This invention is exemplified by a nucleic acid sequence encoding ribosomal protein S18. The activity of S18 promoter is restricted to meristems (Lijsebettesn *et al.*, *EMBO J.* 13: 3378-3388). Transient expression of a nucleic acid sequence in a host plant can trigger a signal transmitting

5   to meristems and affect the gene expression in meristem.

One problem with gene silencing in a plant host is that many plant genes exist in multigene families. Therefore, effective silencing of a gene function may be especially problematic. According to the present invention, however, nucleic acids may be inserted into the viral genome to effectively silence a particular gene function

10   or to silence the function of a multigene family. It is presently believed that about 20% of plant genes exist in multigene families.

A detailed discussion of some aspects of the "gene silencing" effect is provided in U.S. Patent No. 5,922,602; the disclosure of which is incorporated herein by reference. RNA can reduce the expression of a target gene through inhibitory

15   RNA interactions with target mRNA that occur in the cytoplasm and/or the nucleus of a cell.

An EST/cDNA library from a plant such as *Arabidopsis thaliana* may be assembled into a plant viral transcription plasmid background. The cDNA sequences in the transcription plasmid library can then be introduced into plant cells as

20   cytoplasmic RNA in order to post-transcriptionally silence the endogenous genes. The EST/cDNA sequences may be introduced into the plant viral transcription plasmid in either the plus or anti-sense orientation (or both), and the orientation can be either directed or random based on the cloning strategy. A high-throughput, automated cloning strategy using robotics can be used to assemble the library. The

25   EST clones can be inserted behind a duplicated subgenomic promoter such that they are represented as subgenomic transcripts during viral replication in plant cells. Alternatively, the EST/cDNA sequences can be inserted into the genomic RNA of a plant viral vector such that they are represented as genomic RNA during the viral replication in plant cells. The library of EST clones is then transcribed into infectious

30   RNAs and inoculated onto a host plant susceptible to viral infection. The viral RNAs containing the EST/cDNA sequences contributed from the original library are now present in a sufficiently high concentration in the cytoplasm of host plant cells such that they cause post-transcriptional gene silencing of the endogenous gene in a host

plant. Since the replication mechanism of the virus produces both sense and antisense RNA sequences, the orientation of the EST/cDNA insert is normally irrelevant in terms of producing the desired phenotype in the host plant.

5   Genomic libraries containing sequences from rice, barley, corn, soybean and other important crops can be obtained from public and private sources, or be prepared from plant genomic DNAs. BAC clones containing entire plant genomes have been constructed and organized in a minimal overlapping order. Individual BACs are sheared to fragments and directly cloned into viral vectors. Clones that completely cover an entire BAC form a BAC viral vector sublibrary. Genomic clones can be identified by probing

10   filters containing BACs with labeled nucleic acid inserts which result in changes in a host plant, or with labeled probes prepared from DNAs encoding the gene of interest of any organism that has already been cloned. Useful labels include radioactive, fluorescent, or chemiluminecent molecules, enzymes, etc. BACs that hybridize to the probe are selected and their corresponding BAC viral vectors are used to produce infectious RNAs. Plants

15   that are transfected with the BAC sublibrary are screened for change of function, for example, change of growth rate or change of color. Once the change of function is observed, the inserts from these clones or their corresponding plasmid DNAs are characterized by dideoxy sequencing. This provides a rapid method to obtain the genomic sequence for a plant protein.

20   The recombinant nucleic acids and recombinant viruses used in the present invention are constructed using techniques well known in the art. Suitable techniques have been described in Sambrook *et al.*, *Molecular Cloning* (2d ed.), Cold Spring Harbor Laboratory, Cold Spring Harbor (1982, 1989); *Methods in Enzymol.* (Vols. 68, 100, 101, 118, and 152-155) (1979, 1983, 1986, and 1987); *DNA Cloning*, D.M.

25   Clover, Ed., IRL Press, Oxford (1985); and, *Principles of Gene Manipulation* (5th ed.), R.W. Old *et al.*, Blackwell Science, Oxford (1994). Medium compositions have been described by Miller, J., *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, New York (1972), as well as the references previo0usly identified, all of which are incorporated herein by reference. DNA manipulations and enzyme

30   treatments are carried out in accordance with manufacturers' recommended procedures in making such constructs.

In order to provide an even clearer and more consistent understanding of the specification and the claims, including the scope given herein to such terms, the following definitions are provided:

Anti-Sense Inhibition: A type of gene regulation based on cytoplasmic, nuclear or organelle inhibition of gene expression due to the presence in a cell of an RNA molecule complementary to at least a portion of the mRNA being translated. It is specifically contemplated that RNA molecules may be from either an RNA virus or mRNA from the host cells genome or from a DNA virus.

Cell Culture: A proliferating group of cells which may be in either an undifferentiated or differentiated state, growing contiguously or non-contiguously.

Chimeric Sequence or Gene: A nucleotide sequence derived from at least two heterologous parts. The sequence may comprise DNA or RNA.

Coding Sequence: A deoxyribonucleotide or ribonucleotide sequence which, when either transcribed and translated or simply translated, results in the formation of a cellular polypeptide or a ribonucleotide sequence which, when translated, results in the formation of a cellular polypeptide.

Compatible: The capability of operating with other components of a system. A vector or plant or animal viral nucleic acid which is compatible with a host is one which is capable of replicating in that host. A coat protein which is compatible with a viral nucleotide sequence is one capable of encapsidating that viral sequence.

Complementation Analysis: As used herein, this term refers to observing the changes produced in an organism when a nucleic acid sequence is introduced into that organism after a selected gene has been deleted or mutated so that it no longer functions fully in its normal role. A complementary gene to the deleted or mutated gene can restore the genetic phenotype of the selected gene.

Dual Heterologous Subgenomic Promoter Expression System (DHSPES): a plus stranded RNA vector having a dual heterologous subgenomic promoter expression system to increase, decrease, or change the expression of proteins, peptides or RNAs, preferably those described in U.S. Patent Nos. 5,316,931, 5,811,653, 5,589,367, and 5,866,785, the disclosure of which is incorporated herein by reference.

Expressed sequence tags (ESTs): Relatively short single-pass DNA sequences obtained from one or more ends of cDNA clones and RNA derived therefrom. They

may be present in either the 5' or the 3' orientation. ESTs have been shown useful for identifying particular genes.

Expression: The term as used herein is meant to incorporate one or more of transcription, reverse transcription and translation.

5   A Functional Gene Profile: The collection of genes of an organism which code for a biochemical or phenotypic trait. The functional gene profile of an organism is found by screening nucleic acid sequences from a donor organism by over expression or suppression of a gene in a host organism. A functional gene profile requires a collection or library of nucleic acid sequences from a donor organism. A

10   functional gene profile will depend on the ability of the collection or library of donor nucleic acids to cause over-expression or suppression in the host organism. Therefore, a functional gene profile will depend upon the quantity of donor genes capable of causing over-expression or suppression of host genes or of being expressed in the host organism in the absence of a homologous host gene.

15   Gene: A discrete nucleic acid sequence responsible for producing one or more cellular products and/or performing one or more intercellular or intracellular functions.

Gene silencing: A reduction in gene expression. A viral vector expressing gene sequences from a host may induce gene silencing of homologous gene

20   sequences.

Homology: A degree of nucleic acid similarity in all or some portions of a gene sequence sufficient to result in gene suppression when the nucleic acid sequence is delivered in the antisense orientation.

Host: A cell, tissue or organism capable of replicating a nucleic acid such as a

25   vector or viral nucleic acid and which is capable of being infected by a virus containing the viral vector or viral nucleic acid. This term is intended to include prokaryotic and eukaryotic cells, organs, tissues or organisms, where appropriate. Bacteria, fungi, yeast, and animal (cell, tissues, or organisms), are examples of a host.

Infection: The ability of a virus to transfer its nucleic acid to a host or

30   introduce a viral nucleic acid into a host, wherein the viral nucleic acid is replicated, viral proteins are synthesized, and new viral particles assembled. In this context, the terms "transmissible" and "infective" are used interchangeably herein. The term is

also meant to include the ability of a selected nucleic acid sequence to integrate into a genome, chromosome or gene of a target organism.

Insert: a stretch of nucleic acid sequence, typically more than 20 base pairs long.

5   Multigene family: A set of genes descended by duplication and variation from some ancestral gene. Such genes may be clustered together on the same chromosome or dispersed on different chromosomes. Examples of multigene families include those which encode the histones, hemoglobins, immunoglobulins, histocompatibility antigens, actions, tubulins, keratins, collagens, heat shock proteins, salivary glue

10 proteins, chorion proteins, cuticle proteins, yolk proteins, and phaseolins.

Non-Native: Any RNA or DNA sequence that does not normally occur in the cell or organism in which it is placed. Examples include recombinant viral nucleic acids and genes or ESTs contained therein. That is, an RNA or DNA sequence may be non-native with respect to a viral nucleic acid. Such an RNA or DNA sequence

15 would not naturally occur in the viral nucleic acid. Also, an RNA or DNA sequence may be non-native with respect to a host organism. That is, such a RNA or DNA sequence would not naturally occur in the host organism.

Nucleic acid: As used herein the term is meant to include any DNA or RNA sequence from the size of one or more nucleotides up to and including a complete

20 gene sequence. The term is intended to encompass all nucleic acids whether naturally occurring in a particular cell or organism or non-naturally occurring in a particular cell or organism.

Nucleic acid of interest: The term is intended to refer to the nucleic acid sequence whose function is to be determined. The sequence will normally be non-

25 native to a viral vector but may be native or non-native to a host organism.

Phenotypic Trait: An observable, measurable or detectable property resulting from the expression or suppression of a gene or genes.

Plant Cell: The structural and physiological unit of plants, consisting of a protoplast and the cell wall.

30 Plant Organ: A distinct and visibly differentiated part of a plant, such as root, stem, leaf or embryo.

Plant Tissue: Any tissue of a plant in plant or in culture. This term is intended to include a whole plant, plant cell, plant organ, protoplast, cell culture, or any group of plant cells organized into a structural and functional unit.

Positive-sense inhibition: A type of gene regulation based on cytoplasmic
5   inhibition of gene expression due to the presence in a cell of an RNA molecule substantially homologous to at least a portion of the mRNA being translated.

Promoter: The 5'-flanking, non-coding sequence substantially adjacent a coding sequence which is involved in the initiation of transcription of the coding sequence.

10   Protoplast: An isolated plant or bacterial cell without some or all of its cell wall.

Recombinant Viral Nucleic Acid: Viral nucleic acid which has been modified to contain non-native nucleic acid sequences. These non-native nucleic acid sequences may be from any organism or purely synthetic, however, they may also
15   include nucleic acid sequences naturally occurring in the organism into which the recombinant viral nucleic acid is to be introduced.

Recombinant Virus: A virus containing the recombinant viral nucleic acid.

Sense Inhibition: A type of gene regulation based on cytoplasmic, nuclear or organelle inhibition of gene expression due to the presence in a cell of an RNA
20   molecule identical or homologous in sequence to at least a portion of the mRNA being translated. It is specifically contemplated that RNA molecules may be from either an RNA virus or mRNA from the host cells genome or from a DNA virus.

Subgenomic Promoter: A promoter of a subgenomic mRNA of a viral nucleic acid.

25   Substantial Sequence Homology: Denotes nucleotide sequences that are substantially functionally equivalent to one another. Nucleotide differences between such sequences having substantial sequence homology are insignificant in affecting function of the gene products or an RNA coded for by such sequence.

Systemic Infection: Denotes infection throughout a substantial part of an
30   organism including mechanisms of spread other than mere direct cell inoculation but rather including transport from one infected cell to additional cells either nearby or distant.

Targeting Nucleotide Sequence: a nucleotide sequence specifically for targeting the down-regulation or silencing of a gene of interest.

Transient Expression: Expression of a nucleic acid sequence in a host without insertion of the nucleic acid sequence into the host genome, such as by way of a viral

5   vector.

EXAMPLES

The following examples further illustrate the present invention. These examples are intended merely to be illustrative of the present invention and are not to

10   be construed as being limiting.

Biological deposits.

The following strains were deposited with the ATCC, 10801 University Blvd., Manassas, Virginia 20110, U.S.A., on January 18, 2001 (viral vectors are in *E. coli*

15   strain DH5α): DH5α: α.42 (Deposit No.: to be assigned); DH5α: β.42.Sp1(Deposit No.: to be assigned); DH5α: β.Δβa(Deposit No.: to be assigned); DH5α: β.Δβb.GFP (β.GFPc3.Δβb) (Deposit No.: to be assigned); DH5α: β.GFP::βb (β.GFPc3::βb) (Deposit No.: to be assigned); DH5α: β.GFP::2A (β.GFPc3::2A::βb) (Deposit No.: to be assigned); DH5α: γ42(Deposit No.: to be assigned); DH5α: γ.GFP::γb

20   (γ.GFPc3::γb) (Deposit No.: to be assigned); DH5α: γ.GFP::HA::2A::γb (γ.GFPc3::HA::2A::γb) (Deposit No.: to be assigned); DH5α: γ.Δγa.Δγb.GFP (γ.Δγa.Δγb.GFPc3) (Deposit No.: to be assigned); DH5α: γ.Δγb.GFP (γ.Δγb.GFPc3) (Deposit No.: to be assigned); DH5α: γ.bPDS4(γ.γb.stop.bPDS4) (Deposit No.: to be assigned); DH5α: γ.bPDS4-as (γ.γb.stop.bPDS4-as) (Deposit No.: to be assigned);

25   DH5α: γ.Δγa(Deposit No.: to be assigned); and, DH5α: γ.2A::GFP::H (γ.γb::2A::GFP::H) (Deposit No.: to be assigned).

EXAMPLE 1

Expression of heterologous protein fusions from RNA γ.

30   Construction of γ RNA-based monocot viral vectors.

An infectious BSMV RNA gamma (γ) cDNA clone (γ42) (Petty, *et al.* 1989) was modified to express foreign inserts from the γ subgenomic RNA 1, using the γb

subgenomic promoter (sgp) (Figure 1A). The γ42 cDNA was modified to replace nucleotides 5098-5103 with a Nhe I site by site-directed mutagenesis using the oligonucleotide 5' CTTCTTCCGTTGCTAGCTAAAAAAAAAAA 3' (SEQ ID NO: 11) to generate the plasmid γ42.NheI (Figure 1B). This plasmid was further modified

5 to change the MluI plasmid linearization restriction site that immediate follows the viral sequence to one that would not be found in foreign insert sequences. MluI was changed to the 6 base pair (bp) restriction site, BssHII by digesting γ42.NheI with MluI, filling in the overhang with the Klenow fragment of DNA polymerase I, and religating to generate the plasmid γ42.NheI-B (Figure 1B). The BssHII restriction site

10 in γ42.NheI-B was replaced with the 8 base pair restriction site, SwaI, using polymerase chain reaction (PCR) mutagenesis. To achieve this, a 433 bp fragment was amplified from γ42 using the oligonucleotides 5' AGTTACTTCTTGAATTTCTCC 3' (SEQ ID NO: 12) (upstream) and 5' TATAGCGCGC<u>ATTTAAAT</u>TGGTCTTCCCTTGGGGGACCG 3' (SEQ ID NO:

15 13) (downstream). The PCR fragment was digested with Hpa I and Bss HII and inserted between the Hpa I and Bss HII sites of γ42.NheI-B to generate γ42.NheI-S (Figure 1B).

Subsequently, a 646 bp Nhe I fragment, containing the zeomycin resistance gene as a cloning marker, was amplified from pZErO (Invitrogen Corporation,

20 Carlsbad, CA, USA) using PCR mutagenesis with the oligonucleotides
5' TATGCTAGCTGA<u>TTAATTAA</u>GTCGACGAGCTGATTTAACAAATTTTAAC 3'3' (SEQ ID NO: 14) (upstream) and 5'
TATGCTAGCTGA<u>GCGGCCGC</u>GCACGTGTCAGTCCTGCTCCTCGG 3' (SEQ ID NO: 15) (downstream) as primers. This PCR fragment was digested with Nhe I

25 and inserted into the Nhe I site of γ42.NheI-B and γ42.NheI-S to generate four plasmids, γ.γb.stop.P/N.Zeo-B (positive orientation), γ.γb.stop.N/P.Zeo-B (negative orientation) and γ.γb.stop.P/N.Zeo-S (positive orientation), γ.γb.stop.N/P.Zeo-S (negative orientation), respectively (Figure 1C). All four plasmids have PacI and NotI sites flanking the zeomycin resistance gene and contain a stop codon at the end of the

30 γb ORF to prevent translation of downstream sequences.

The BSMV γ42.NheI-S cDNA was modified to express GFPc3 as a fusion to the C-terminus of the BSMV γb protein in either the sense and antisense orientation.

Using PCR mutagenesis a 764 bp fragment, containing the green fluorescent protein

cycle 3 (GFPc3) open reading frame (ORF) (Crameri, *et al.*, 1996), was amplified

from TMV-SBS5::GFPc3 using the oligonucleotides

5' TATACTAGT<u>TTAATTAA</u>GTCGACCATGGCTAGCAAAGGAGAAGAAC 3'

5   (SEQ ID NO: 16) (upstream) and

5' TATACTAGTTGA<u>GCGGCCGC</u>TTATTTGTAGAGCTCATCCATGCC 3' (SEQ

ID NO: 17) (downstream). The PCR fragment was digested with Spe I and inserted

into the compatible Nhe I site of γ42.NheI-S. This generated two

plasmids, γ.γb::GFPc3 (positive orientation) and γ.γb.stop.GFPc3-as (negative

10   orientation), with PacI and NotI sites flanking the GFPc3 gene (Figure 2A). The

plasmid γ.γb.stop.GFPc3-as, to be used as a negative control, contains a stop codon at

the end of the γb ORF to prevent translation of the downstream antisense GFPc3

sequence.

The BSMV γ42.NheI-S cDNA was modified to express GFPc3 as a fusion to

15   the N-terminus of the BSMV γb protein. A 616 bp fragment, containing the BSMV

γb ORF, was amplified from γ42 using PCR and the oligonucleotides

5' TATAGAGCTCTACAAATCTAGAATGGCTACTTTCTCTTGTGTG 3'(SEQ

ID NO: 18) (upstream) and 5' AGAGTCCGTTAAGATTCATGG 3' 3'(SEQ ID NO:

19) (downstream) as primers. The PCR fragment was digested with SacI and PflMI

20   and inserted into the SacI and PflMI sites of γ.Δγb.GFPc3 to generate γ.GFPc3::γb

(Figure 2B).

Inoculation protocol.

Infectious BSMV RNAs from cDNA clones were prepared by *in vitro* transcription

25   using T7 DNA-dependent RNA polymerase (Ambion) as described previously (Petty,

et al; 1989). Transcripts of each of the BSMV genomes (wild type or genetically

modified) were mixed in a 1:1:1 ratio, unless otherwise noted. A 7.5 µl aliquot of the

transcription mix was combined with 45 µL of FES (Pogue, *et al.*, 1998) and directly

applied to 12 day old black hulless barley, or *N. benthamiana* plants. Systemic spread

30   was determined by the appearance of mosaic symptoms and by local lesion assay on

Chenopodium amranticolor. The presence of heterologous inserts in systemic virus

populations was confirmed by Reverse Transcriptase (RT)-PCR using primers specific to the BSMV γ RNA and flanking the inserted cDNA sequence.

Results and discussion.

5   In this approach, BSMV was used to expressed GFPc3, as a fusion to the N-terminus of the γb protein, in tobacco protoplasts and in systemically infected barley. Previously, GFP had been expressed as a fusion to C-terminus of the γb protein in BSMV (Figure 1B, Haupt, *et al.*, 2001, Lawrence and Jackson, submitted to *Mole. Plant Pathol.*, on December 20, 2000). However, the γb::GFP fusion was unstable in

10   the virus as it spread systemically.

     To improve the stability of the GFP insert we designed a BSMV vector to express GFP as a fusion to the N-terminal of the γb protein (GFPc3::γb). High levels of γb::GFPc3 were expressed systemically by BSMV from the γ RNA of BSMV (Figure 2A). As mentioned above, systemic expression of the γb::GFPc3 fusion

15   protein has been demonstrated barley (inoculation number 3, Table 1). The fusion of GFPc3 to C-termini of the γb protein resulted in GFPc3 accumulation in barley to levels within 30-50% of that produced by TMV 30B in *N. benthamiana* (compare inoculation number 2 with 3, Table 1). However, expression is highly variable from plant to plant and RT-PCR experiments using primers flanking the GFP insert

20   revealed that the virus deletes the GFPc3 sequence as it spreads (data not shown). Thus, expression is principally observed in 1-2 leaves above inoculated leaf. In general, the GFPc3 gene is maintained and expressed by the virus for 7-10 dpi, though GFPc3 expression has occasionally been observed up to 25 dpi in the 3rd and 4th leaves above the inoculated leaf (less than 10% of infected plants), or not at all (less

25   than 10% of infected plants).

     An alternative site for gene expression is to fuse foreign gene sequences at the N-terminus of γb gene. The fusion of GFPc3 to N-terminus of the γb protein (GFPc3::γb, Figure 2B) resulted in very high levels of GFPc3 fluorescence and GFPc3::γb accumulation in barley (inoculation number 5, Table 1). However, GFPc3

30   fluorescence and protein accumulation are observed longer and more consistently in BSMV-GFPc3::γb infected plants than in those infected with BSMV-γb::GFPc3. For example, GFPc3::γb expression is observed in 3-4 leaves above the inoculated leaf by

BSMV-GFP::γb, compared to 1-2 leaves for BSMV-γb::GFPc3. Furthermore, the GFPc3 gene is generally maintained and expressed by BSMV::GFPc3::γb to 18 dpi, and can often be observed at 25 dpi in the 5th leaf above the inoculated leaf and in the axillary shoots (about 30% of the infected plants).

5   In conclusion, our results demonstrate that fusions of heterologous proteins to the BSMV γb protein can be systemically expressed in a monocot, and that fusions to N-terminus of the γb protein are more stable than to the C-terminus.

Table 1*

Inoc. No.	Test Constructs 1	Test Constructs 2	α	β	γ	Tobacco Protoplasts	Barley Plants (GFP expression)	Barley Plants (Mosaic Phenotype)
1	none		X	X	X			heavy
2	TMV-SBS5 c3					*****		
3	γ.γb::GFPc3		X	X		****	systemic	heavy
4	γ.γb::2A::GFPc3		X	X		****	systemic	heavy
5	γ.GFPc3::γb		X	X		nt	systemic	heavy
6	γ.GFPc3::2A::γb		X	X		nt	systemic	heavy
7	γ.Δγb		X	X				light
8	γ.Δγb.GFPc3		X	X		**		
9	γ.GFPc3.stop.γb		X			nt		moderate
10	γ.Δγb.GFPc3		X	X	X	***		heavy
11		β.γb::βb	X		X	nt		
12	γ.Δγb	β.γb::βb	X			nt		
13	γ.Δγb.GFPc3	β.γb::βb	X			nt		
14		β.γb::mt2A::βb	X		X	nt		
15	γ.Δγb.1	β.γb::mt2A::βb	X					
16	γ.Δγb.GFPc3	β.γb::mt2A::βb	X			**		
17		β.γb::2A::βb	X		X	nt		heavy
18	γ.Δγb.1	β.γb::2A::βb	X					heavy/mod.
19	γ.Δγb.GFPc3	β.γb::2A::βb	X			**		
20		β.Δβa	X		X			heavy
21	γ.γb::GFPc3	β.Δβa	X			*****	bright systemic	heavy
22	γ.γb::2A::GFPc3	β.Δβa	X			nt	nt	nt
23	γ.GFPc3::γb	β.Δβa	X			nt	bright systemic	heavy
24	γ.GFPc3::2A::γb	β.Δβa	X			nt	bright systemic	heavy
25	γ.Δγb.GFPc3	β.Δβa	X			***		
26	γ.Δγb.GFPc3	β.Δβa	X		X	****		heavy

Explanation of abbreviation/symbols: "nt": not tested; under the "Tobacco protoplasts" column (expression is measured against the expression of TMV-SBS5::GFPc3 in tobacco protoplasts): "" is 0 to less than 1% (low expression), "**" is 1 to less than 5% (moderate expression), "***" is 5 to less than 10% (moderately high expression), "****" is 10 to less than 100% expression (high expression), "*****" is 100 to less than 200% (very high expression), "******" is 200 to less than 500% (very high expression), and "*******" is 500% or more (very high expression); under the "Barley Plants" column: "systemic" is visual detection of systemic GFP expression, "bright systemic" is visual detection of very bright systemic GFP expression; under the "Mosaic Phenotype" column: "light" is about 25% of the plant surface displaying symptomatic tissue, "moderate" and "heavy/moderate" is about 25-50% of the plant surface displaying symptomatic tissue, "heavy" is about 50-75% of the plant surface displaying symptomatic tissue.

EXAMPLE 2

Expression of native heterologous proteins from RNA γ derived vectors.

Construction of monocot viral vectors.

5   The BSMV vectors expressing N- and C-terminus fusions of GFPc3 to BSMV

γb were modified to release GFPc3 after *in vivo* cleavage of the fusion protein.

Cleavage of the fusion protein was achieved by inserting the foot and mouth disease

virus (FMDV) 2A autoproteolytic sequence (Ryan, *et al.*, 1991) between the fused

ORFs. To construct a vector expressing GFPc3::2A::γb, a 824 bp fragment,

10   containing the GFPc3 ORF, was amplified from the TMV-SBS5::GFPc3 cDNA using

PCR and the oligonucleotides

5' CATTAATTAAGATGATGGCTAGCAAAGGAG 3'(SEQ ID NO: 20)

(upstream) and 5'

ATATCTAGACCTAGGACCAGGGTTAGATTCCACGTCACCCGCCAACTTCA

15   GCAAATCAAAATTCAACAGCTGTTTGTAGAGCTCAGCGGCCGCCTTGTAT

AGCTCATCCATG 3' (SEQ ID NO: 21) (downstream) as primers. The PCR

fragment was digested with SacI and inserted into γ.GFPc3::γb treated sequentially

with SphI, Mung bean nuclease and SacI to generate γ.GFPc3::2A::γb (Figure 2C). To

construct a vector expressing γb::2A::GFPc3, a 818 bp fragment, containing the

20   GFPc3 ORF, was amplified from the TMV-SBS5::GFPc3 cDNA using PCR and the

oligonucleotides 5'

TATACTAGTCAGCTGTTGAATTTTGATTTGCTGAAGTTGGCGGGTGACGTG

GAATCTAACCCTGGTCCTGTCGACAAAGGAGAAGAACTTTTCACTGG

3'(SEQ ID NO: 22) (upstream) and 5'

25   TATGCTAGCGATCAATTAGCGGCCGCTTATTTGTAGAGCTCATCCATGC

3'(SEQ ID NO: 23)(downstream) as primers. The PCR fragment was digested with

SpeI and NheI and inserted into the compatible NheI site of BSMV γ42.NheI-S to

generate γ.γb::2A::GFPc3 (Figure 2C).

30   Inoculation protocol.

          See Example 1.

Results and discussion.

For many applications it is desirable to produce heterologous proteins in monocot plants that are not fused to virus proteins. For example, many proteins require precise cellular localization to be functional, and fusion of a heterologous

5   protein to a viral protein can prevent proper localization of either the heterologous protein, or the viral protein. In addition, fusion of one protein to another can inhibit activity of either protein due to improper folding or steric interference in a manner which is difficult to predict. To avoid these problems, the FMDV 2A translational cleavage sequence has been inserted into the both of the BSMV vectors described in

10   Example 1, between γb ORF and that of GFPc3 (Figure 2C). The FMDV 2A cleavage sequence is 90-100% effective in both the N-terminal fusion of GFPc3 to γb (GFPc3::2A::γb) and the C-terminal fusion (γb::2A::GFPc3) as judged by Western analysis of product from infected tobacco protoplasts and barley plants (data not shown). Viral spread and GFPc3 fluorescence in barley plants infected with BSMV-

15   GFPc3::2A::γb are indistinguishable from those containing BSMV-GFPc3::γb. In contrast, the systemic spread of the BSMV-γb::2A::GFPc3 virus is slightly delayed compared to BSMV-γb::GFPc3. Both BSMV-γb::2A::GFPc3 and BSMV-GFPc3::2A::γb are able to infect and express GFPc3 in *N. benthamiana* plants in a manner similar to that reported for BSMV-γb::GFP (Lawrence and Jackson, submitted

20   to *Mole. Plant Pathol.*, submitted on December 20, 2000).

EXAMPLE 3

Expression of epitope tagged heterologous proteins from RNA γ derived vectors.

25   Construction of monocot viral vectors.

The BSMV vector γ.GFPc3::2A::γb was modified to express epitope tagged GFPc3 fused to the N-terminus of the FMDV 2A cleavage sequence and BSMV γb protein. Epitope tagging of GFPc3 was achieved by inserting either the hemagglutinin (HA), c-Myc epitope, or 9x poly-histidine epitope tag between GFPc3 ORF and the

30   FMDV 2A/BSMV γb fusion ORF. To construct a vector expressing GFPc3::HA::2A::γb, a linker consisting of the oligonucleotides

5' GGCCGCTTATCCGTATGATGTTCCGGATTATGCCGAGCT 3' (SEQ ID NO: 24)(and 5' CGGCATAATCCGGAACATCATACGGATAAGC 3' (SEQ ID NO: 25) was inserted into the NotI and SacI sites of γ.GFPc3::2A::γb, generating γ.GFPc3::HA::2A::γb (Figure 2D). To construct a vector expressing

5   GFPc3::Myc::2A::γb, a linker consisting of the oligonucleotides
5' GGCCGCTGAACAAAAGCTTATCTCTGAGGAAGATCTTGAGCT 3' (SEQ ID NO: 26) and 5' CAAGATCTTCCTCAGAGATAAGCTTTTGTTCAGC 3' 3' (SEQ ID NO: 27) was inserted into the NotI and SacI sites of γ.GFPc3::2A::γb, generating γ.GFPc3::Myc::2A::γb (Figure 2D). To construct a vector expressing

10 GFPc3::H::2A::γb, a linker consisting of the oligonucleotides
5' GGCCGCTCATCATCACCATCACCATCACCATCACGAGCT 3' (SEQ ID NO: 28) and 5' CGTGATGGTGATGGTGATGGTGATGATGAGC 3'(SEQ ID NO: 29) was inserted into the NotI and SacI sites of γ.GFPc3::2A::γb, generating γ.GFPc3::H::2A::γb (Figure 2D).

15   The BSMV vector γ.GFPc3::H::2A::γb was modified to express five selected proteins as poly-histidine tagged fusions to the N-terminus of the FMDV 2A cleavage sequence and BSMV γb protein. To construct a vector expressing the brome mosaic virus coat protein (BMV-CP, Ahlquist, *et al.*, 1981), the oligonucleotides
5' TATTTAATTAAGATGTCGACTTCAGGAACTGG 3' (SEQ ID NO: 30)

20 (upstream) and 5' TATGCGGCCGCCCTATAAAGCGGGGTGAAG 3' (SEQ ID NO: 31) (downstream) were used to amplify a 593 bp fragment from 30B-BMVcp 1. This fragment, containing the BMV cp ORF, was digested with PacI and NotI and inserted between the PacI and NotI sites of γ.GFPc3::H::2A::γb, replacing the GFPc3 ORF and generating γ.BMCcp::H::2A::γb. To construct a vector expressing chicken

25 interferon (chIF, Digby and Lowenthal, 1995), the oligonucleotides
5' TATTTAATTAAGATGACTTGCCAGACTTACAAC 3' (SEQ ID NO: 32) (upstream) and 5' TATGCGGCCGCGCAATTGCATCTCCTCTGAG 3'(SEQ ID NO: 33) (downstream) were used to amplify a 518 bp fragment from 30B-chINF. This fragment, containing the chIF ORF, was digested with PacI and NotI and

30 inserted between the PacI and NotI sites of γ.GFPc3::H::2A::γb, replacing the GFPc3 ORF and generating γ.chIF::H::2A::γb. To construct a vector expressing bovine lysozyme (boLys, Irwin and Wilson, 1989), the oligonucleotides

5' TATTTAATTAAGATGAAGGCTCTCGTTATTCTGG 3' (SEQ ID NO: 34)
(upstream) and 5' TATGCGGCCGCCAGGGTGCAACCCTCAACG 3' (SEQ ID
NO: 35) (downstream) were used to amplify a 467 bp fragment from 735 bolys. This
fragment, containing the boLys ORF, was digested with PacI and NotI and inserted

5   between the PacI and NotI sites of γ.GFPc3::H::2A::γb, replacing the GFPc3 ORF and
generating γ.boLys::H::2A::γb. To construct a vector expressing human growth
hormone (hGH, DeNoto, *et al.*, 1981), the oligonucleotides
5' TATTTAATTAAGATGGGAAAAATGGCTTCTCTATTTGC 3' (SEQ ID NO:
36) (upstream) and 5' TATGCGGCCGCGAAACCGCAGGAACCTTCAACG

10   3'(SEQ ID NO: 37) (downstream) were used to amplify a 677 bp fragment from 30B-
(HCPRO)-hGH. This fragment, containing the hGH ORF, was digested with PacI and
NotI and inserted between the PacI and NotI sites of γ.GFPc3::H::2A::γb, replacing
the GFPc3 ORF and generating γ.hGH::H::2A::γb. To construct a vector expressing
tomato protease inhibitor 1 (tPI1, Graham, *et al.,* 1985), the oligonucleotides

15   5' TATTTAATTAAGATGGAGTCAAAGTTTGCTCAC 3' (SEQ ID NO: 38)
(upstream) and 5' TATGCGGCCGCAGTCACCACAGGCATTGTAC 3' (SEQ ID
NO: 39) (downstream) were used to amplify a 359 bp fragment from 735-tomPI 3.
This fragment, containing the tPI1 ORF, was digested with PacI and NotI and inserted
between the PacI and NotI sites of γ.GFPc3::H::2A::γb, replacing the GFPc3 ORF and

20   generating γ.tPI::H::2A::γb.

Inoculation protocol.
        See Example 1.

25 ·   Results and discussion.
        Many applications require the ability to detect and be able to isolate a protein.
Epitope tags provide a convenient method for both detection and purification of
proteins because they are small protein sequences that are recognized by
commercially available antibodies (Jarvik and Telmer, 1998). To detect heterologous

30   proteins expressed by the BSMV vector in Example 2, epitope tags were inserted
between GFPc3 and the 2A sequence of the GFP::2A::γb fusion to generate three
different vectors (Figure 2D). Each epitope tagged GFPc3 was analyzed by Western

blotting and probed with the available antibodies. Both the anti-HA (anti-hemagglutinin, Y-11 and F-7 Santa Cruz Biotechnology, Santa Cruz, California) and anti-Myc (anti c-Myc, A-14 and 9E10, Santa Cruz Biotechnology, Santa Cruz, California) antibodies were able to efficiently detect their respective tags when

5   compared to the anti-GFP antibody. However, the H (poly-histidine) tag was only weakly recognized by the anti-histidine antibody (H-15, Santa Cruz Biotechnology, Santa Cruz, California). The addition of coding sequences for the HA and Myc peptide tags will allow the efficient detection of expression of non-processed proteins expressed from virus vectors. The only exception would be for a subset of vacuolar

10   proteins that are proteolytically processed to remove a C-terminal vacuolar sorting sequence (VSS) (reviewed in Chrispeels and Raikhel, *Plant Physiol.* 122:1-2 (2000)).

Five proteins besides GFPc3 were expressed as N-terminal fusions to H::2A::γb (Figure 2D). For each protein expressed, systemically infected tissue was harvested at 6 dpi and compared to local leaves from *N. benthamiana* infected with

15   TMV expressing the same protein. Crude protein extracts were subjected to Western blotting using cognate antibodies raised against the heterologous protein of interest (Figure 3A-E). Since the H epitope tag and the 2A sequence remain on the heterologous protein following cleavage from γb, these proteins are 4 kD larger when expressed by BSMV than those expressed by the TMV vector. In each case, the size

20   of the protein detected in the western blot was consistent with the size predicted for the post-cleavage heterologous protein. The brome mosaic virus coat protein (BMV-CP, Ahlquist, *et al.*, 1981) and chicken interferon (chIF, Digby and Lowenthal, 1995) expressed from BSMV accumulated to about 75% that of a TMV vector in *N. benthamiana* (Figure 3A, 3B). Bovine lysozyme (boLys, Irwin and Wilson, 1989),

25   human growth hormone (hGH, DeNoto, et al., 1981), and tomato protease inhibitor 1 (tPI1, ref) were also expressed successfully from BSMV, though at apparently lower levels, relative to a TMV in *N. benthamiana* (Figure 3C-E).

Four of the proteins, bovine lysozyme, chicken interferon, human growth hormone, and protease inhibitor, contain N-terminal signal peptides (SP) which target

30   the proteins to the endoplasmic reticulum (ER) while they are translated by the ribosome. For proteins targeted to the ER, the SP is removed, and the remainder of the protein is transported to its ultimate destination. In the case of bovine lysozyme, chicken interferon, human growth hormone and protease inhibitor, this destination is

lysozyme, chicken interferon, human growth hormone and protease inhibitor, this destination is the apoplast. The 2A autoproteolytic peptide allows these protiens to reach their normal destination without interefering with the cytoplasmic destination of the γb protein to which they are fused. This occurs because 2A mediated cleavage

5 occurs cotranslationally (Ryan, *et al.*, 1999), after allowing the N-terminal, ER targeted protein to enter the ER, but before the γb is targeted. Expression of bovine lysozyme, chicken interferon, human growth hormone, and protease inhibitor by BSMV as 2A fusions to the N-terminal of γb produces proteins of the size expected after ER targeting and removal of the SP (Figures 3B-E). Since the viruses expressing

10 these proteins are as infectious as the wild-type virus, it can be inferred that the γb protein is being cytoplasmically targeted.

The flexibility of the BSMV expression vector to successfully express proteins of cytosolic localization (GFP and BMV coat protein) and those that are matured through the plant secretory pathway (bovine lysozyme, chicken interferon, human

15 growth hormone and protease inhibitor). These data also demonstrate the ability of the vector to express genes of both plant and animal sources successfully.

EXAMPLE 4

Heterologous protein substitution of BSMV γb ORF.

20

Construction of monocot viral vectors.

The BSMV γ42.NheI-S cDNA was modified to express GFPc3 in place of the BSMV γb protein. First, the γb ORF was removed by using PCR mutagenesis. Oligonucleotides 5' ATAGATATCGATCCCCTTATAGTGC 3' (SEQ ID NO: 40)

25 (upstream) and 5' ATAGCTAGCAAGCATGCGAAGGTAAATACAGTAG 3' (SEQ ID NO: 41) (downstream) were used to amplify a 200 bp fragment from γ42, containing the 185 nucleotides immediately upstream of the γb ORF followed by a modified start codon containing an Sph I restriction site. This PCR fragment was digested with EcoRV and NheI and inserted between the downstream EcoRV site and

30 the NheI site of BSMV γ42.NheI-S cDNA to generate γ.Δγb (Figure 2E, 5B). Subsequently, GFPc3 was amplified from 30B::GFPc3 using the oligonucleotides 5' TATACTAGTTTAATTAAGTCGACCATGGCTAGCAAAGGAGAAGAAC 3'

(SEQ ID NO: 42) (upstream) and 5'
TATTCTAGATGA<u>GCGGCCGC</u>TTATTTGTAGAGCTCATCCATGCC 3' (SEQ ID
NO: 43) (downstream). The PCR fragment was digested with NheI and XbaI and
inserted into the compatible NheI site of γ.Δγb to generate γ.Δγb.GFPc3 (Figure 2E,

5   5C).

The BSMV γ42.NheI-S cDNA was modified to express GFPc3 from the γb
subgenomic RNA but without expression of the γb ORF. A 619 bp fragment,
containing the BSMV γb ORF, was amplified from γ42 using PCR and the
oligonucleotides

10   5' TATAGAGCTCTACAAA<u>TAA</u>TCTAGAATGGCTACTTTCTCTTGTGTG 3'
(SEQ ID NO: 44) (upstream) and 5' AGAGTCCGTTAAGATTCATGG 3' (SEQ ID
NO: 45) (downstream) as primers. The PCR fragment was digested with SacI and
PflMI and inserted into the SacI and PflMI sites of γ.Δγb.GFPc3 to generate
γ.GFPc3.stop.γb (Figure 2E). γ.GFPc3.stop.γb has a stop codon at the end of the

15   GFPc3 ORF that prevents the γb ORF from being translated.

The BSMV β42SpI cDNA was modified to express the BSMV γb protein as
an N-terminal fusion to the βb protein. A 487 bp fragment, containing the BSMV γb
ORF, was amplified from BSMV γ42 using PCR and the oligonucleotides 5'
ATATAGGTCTCCCATGATGGCTACTTTCTCTTGTG 3' (SEQ ID NO: 46)

20   (upstream) and 5' TATTAGGTCTCCCATGGCCTTAGAAACGGAAGAAGAATC
3' (SEQ ID NO: 47) (downstream) as primers. The PCR fragment was digested with
BsaI and inserted into the compatible NcoI site of β42SpI to generate β.γb::βb (Figure
4A).

The BSMV β RNA vector expressing the BSMV γb protein as an N-terminal

25   fusion to the βb protein was modified to release γb after *in vivo* cleavage of the fusion
protein. Cleavage of the fusion protein was achieved by inserting the FMDV 2A
autoproteolytic sequence between the fused ORFs. A 547 bp fragment, containing the
BSMV γb ORF, was amplified from γ.γb::2A::GFPc3 using PCR and the
oligonucleotides 5' ATATAGGTCTCCCATGATGGCTACTTTCTCTTGTG 3'

30   (SEQ ID NO: 48) (upstream) and 5'
TATTAGGTCTCCCATGGCAGGACCAGGGTTAGATTCC 3' (SEQ ID NO: 49)

(downstream) as primers. The PCR fragment was digested with BsaI and inserted into the compatible Nco I site of β42SpI to generate β.γb::2A::βb (Figure 4A).

Inoculation protocol.

5   See Example 1.

Results and discussion.

          Another strategy to produce heterologous proteins without fusion to viral proteins was to replace the γb ORF with that of the heterologous ORF. Substitution of

10   the GFPc3 ORF for the γb ORF (Figure 2E) resulted in moderate GFPc3 expression in protoplasts, but the virus was not detected, and GFPc3 was not observed, *in planta* (inoculation number 8, Table 1). The lack of systemic movement of the γ.Δγb.GFPc3 virus was unexpected, since viruses bearing γb deletions (γ.Δγb, Figure 2E) are capable of systemic spread, albeit more slowly than wild type BSMV (Petty, *et al.*,

15   1990, inoculation number 7, Table 1). There is evidence that RNA sequences within γb may boost infectivity (Zhou, *et al.*, 1996). Inserting all of the deleted γb sequence as an untranslated ORF downstream of the GFPc3 gene (Figure 2E) partially restored infectivity to the virus with γb, but did not confer systemic GFPc3 expression in barley plants (inoculation number 9, Table 1).

20   The results of several protoplast inoculations suggested that the γb substitution vector might produce more GFPc3, and also be systemically infectious, if the γb function were complemented in a three component system. For example, if the γb replacement vector was inoculated with the wt γ RNA as a fourth component GFPc3 accumulation in protoplasts increased 2-5 fold over the three component system

25   (compare inoculation number 8 with 10 and 33 with 34, Table 1). However, neither of these four component systems expressed GFPc3 systemically in barley, apparently due to the loss of the GFPc3 bearing component during systemic movement. Therefore, to achieve γb complementation in a three component system, γb was fused (with and without the 2A sequence) to the N-terminal of the βb protein on the β RNA

30   (Figure 4A). In addition, β.γb.mt2A.βb, a construct with a mutation in the 2A site which prevents cleavage of the fusion protein, was also tested. Without a functional 2A site between the γb::βb fusion, βb activity is lost and the virus is not systemically

infectious (inoculation number 11-16, Table 1). When the functional 2A sequence was included between γb and βb, βb and γb were both functional and the virus could move systemic (inoculation number 17 and 18, Table 1), but not with a γ RNA bearing the GFPc3 substitution of the γb ORF (inoculation number 19, Table 1).

5

EXAMPLE 5

Effect of coat protein deletion heterologous protein expression from RNA γ derived vectors.

10   Construction of monocot viral vectors.

To improve the expression of the γ subgenomic RNA1, an infectious BSMV RNA beta (β) cDNA (β42SpI) (Petty, *et al*; 1989) was modified by deleting the majority of the coat protein ORF by PCR mutagenesis. To generate the deletion, a 423 bp fragment was amplified from β42SpI using the oligonucleotides 5'

15   GGAAAGCCGGCGAACGTGGCG 3' (SEQ ID NO: 50) (upstream) and 5' TATATTCGAATCTAGAATCGATGCTAGCTTGCATGCTGTGAAGTGGTAAAA GAAATGC 3' (SEQ ID NO: 51) (downstream) and cloned into the NgoMIV and BstBI sites of β42SpI, creating plasmid β.Δβa (Figure 4B). This construct contains only an untranslatable portion of the coat protein ORF that is required for expression

20   of the subsequent β RNA ORFs.

Inoculation protocol.
        See Example 1.

25   Results and discussion.

The coat protein (βa) of BSMV is not required for systemic infection. To determine the impact of βa deletion on γb expression, a β genomic RNA containing a deletion of the βa ORF was constructed (β.Δβa, Figure 4B). In tobacco protoplasts, using β.Δβa increased GFPc3 accumulation 5 to 10 fold for viruses bearing either the

30   γb::GFPc3 fusion or GFPc3 substitution of γb (compare inoculation number 3 with 21, 8 with 25, and 10 with 26, Table 1). Using β.Δβa also increased GFPc3 accumulation

for γb::GFPc3, GFPc3::γb, and GFPc3::2A::γb, in systemic barley tissue (compare inoculation number 3 with 21, 5 with 23, and 6 with 23, Table 1).

EXAMPLE 6

5   Expression of Heterologous proteins from BSMV RNA β.

Construction of viral vectors.

The BSMV β42SpI cDNA was modified to express GFPc3 as an N-terminal fusion to the βb protein. A 757 bp fragment, containing the GFPc3 ORF, was

10   amplified from the TMV-SBS5::GFPc3 cDNA using PCR and the oligonucleotides 5'
ATATAGGTCTCCCATGGCTAGCAAAGGAGAAGAAC 3' (SEQ ID NO: 52)
(upstream) and 5'
TATTAGGTCTCACATGCATGCTCTAGATTTGTAGAGCTCATCCATGCC 3'
(SEQ ID NO: 53) (downstream) as primers. The PCR fragment was digested with

15   BsaI and inserted into the compatible NcoI site of β42SpI to generate β.GFPc3::βb
(Figure 4C).

The BSMV β RNA vector expressing GFPc3 as an N-terminal fusion to the βb protein was modified to release GFPc3 after *in vivo* cleavage of the fusion protein. Cleavage of the fusion protein was achieved by inserting the FMDV 2A

20   autoproteolytic sequence between the fused ORFs. An 809 bp fragment, containing the GFPc3 ORF, was amplified from the TMV-SBS5::GFPc3 cDNA using PCR and the oligonucleotides 5' ATATAGGTCTCCCATGGCTAGCAAAGGAGAAGAAC
3' (SEQ ID NO: 54) (upstream) and 5'
TTAGGTCTCACATGTCTAGAGGACCAGGGTTAGATTCCACGTCACCCGCC

25   AACTTCAGCAAATCAAAATTCAACAGCTGTTTGTAGAGCTCATCCATGCC
3' (SEQ ID NO: 55) (downstream) as primers. The PCR fragment was digested with BsaI and inserted into the compatible NcoI site of β42SpI to generate
β.GFPc3::2A::βb (Figure 4C).

30   Inoculation protocol.

See Example 1.

Results and discussion.

In this strategy, genetic modifications made to the BSMV β RNA allow it to express the green fluorescent protein (GFP) during infections with the other BSMV genomic RNAs, α and γ, in the monocot host, barley. Moderate, to high, levels of
5   GFPc3 were expressed systemically by BSMV from the β RNA of BSMV as a fusion to the N-terminal of the βb protein (Figure 4C). The fusion of GFPc3 to the N-terminal of the βb resulted in GFPc3 accumulation in barley to levels within 30-50% of that produced by TMV 30B in *N. benthamiana*. This is similar to the amount of GFPc3 produced by the γb::GFP fusion (compare inoculation number 3 and 5, Table
10   2). The extensiveness of GFPc3 expression was improved by 2A-mediated cleavage and release from the GFPc3::2A::βb fusion protein (Figure 4C, inoculation number 6, Table 2) but not by a similar virus with a mutation that deactivates the 2A activity (inoculation number 7, Table 2). Although the GFPc3 gene can be maintained by the GFPc3::βb or GFPc3::2A::βb viruses to 14 dpi, both viruses move more slowly than
15   GFPc3 fusions to γb. Therefore, the net duration and extent of systemic GFPc3 expression by GFP::βb and GFP::2A::βb is somewhat less that of the γb fusions. These results suggest that a BSMV vector based on heterologous protein fusions to the N-terminal of the βb (inoculation number 6, Table 2) and γb (inoculation number 24, Table 1) may be useful for the expression of two or more genes simultaneously in
20   systemic barley tissue.

Both the GFP::βb or GFP::2A::βb viruses expressed 100-500 fold lower amounts of GFPc3 in infected tobacco protoplasts relative to GFPc3 fusions to γb, even though the two groups accumulated similar levels in barley plants (compare inoculations number 5-7 with 3 Table 2). This discrepancy between the performances
25   of βb fusions in plants vs. protoplasts may reflect differences in βb subgenomic promoter activity. Whether this discrepancy is due to the behavior of protoplasts vs, plant tissue is unclear or effect of host specific factors (barley vs. tobacco) is not known.

Table 2*

	Inoculum						GFP expression		Mosaic
	Test Constructs		wt RNAs			Tobacco	Barley		Barley
#	1	2	α	β	γ	Protos	Plants		Plants
1	none		X	X	X				heavy
2	TMV-SBS5 GFPc3					*****			
3	γ.γb.GFPc3		X	X		****	systemic		heavy
4	γ.γb.GFPc3	β.Δβa	X			*****	bright systemic		heavy
5	β.GFPc3::βb		X		X	*	systemic		light
6	β.GFPc3::2A::βb		X		X	*	systemic		moderate
7	β.GFPc3::mt2A::βb		X		X	*	systemic		light
8		β.c3.Δβb	X		X	**			
9	γ.γb::2A::βb		X	X		nt			light
10	γ.γb::2A::βb	β.c3.Δβb	X			nt			light
11	γ.γb::βb		X	X		nt	nt		nt
12	γ.γb::βb	β.c3.Δβb	X			nt			
13		β.γb::2A::βb	X		X	nt			heavy
14		β.γb::βb	X		X	nt			
15	γ.GFPc3::γb		X	X		nt	systemic		heavy

*See Table 1 for explanation of abbreviation/symbols.

EXAMPLE 7

Expression of Heterologous proteins from BSMV RNA β.

Construction of viral vectors.

The BSMV β42SpI cDNA was modified to express GFPc3 as a replacement for the βb protein. A 750 bp fragment, containing the GFPc3 ORF, was amplified from the TMV-SBS5::GFPc3 cDNA using PCR and the oligonucleotides 5' ATATAGGTCTCCCATGGCTAGCAAAGGAGAAGAAC 3' (SEQ ID NO: 56) (upstream) and 5' TATTAGAATTCTCTAGATTATTTGTAGAGCTCATCCATGCC 3' (SEQ ID NO: 57) (downstream) as primers. The PCR fragment was digested with BsaI and Eco RI, and inserted between the BsaI and EcoRI sites of β42SpI to generate β.GFPc3.Δβb (Figure 4D).

The BSMV γ42.NheI-S cDNA was modified to express the βb protein as a fusion to the C-terminus of the BSMV γb protein, both with and without the FMDV 2A cleavage sequence. Using PCR mutagenesis, a 1557 bp fragment, containing the βb protein ORF, was amplified from β42SpI using the oligonucleotides 5'

5   TATACTAGTATGGACATGACGAAAACTGTTG 3' (SEQ ID NO: 58) (upstream) and 5' TATGCTAGCTTATTTGGCCTTGAACCAACTG 3' (SEQ ID NO: 59) (downstream). The PCR fragment was digested with NheI and SpeI and inserted into the compatible NheI site of γ42.NheI-S to generate γ.γb::βb (Figure 4E). Using PCR mutagenesis, a 1623 bp fragment, containing the 2A::βb protein ORF, was amplified

10   from βGFPc3::2A::βb using the oligonucleotides 5' TATACTAGTCAGCTGTTGAATTTTGATTTGC 3' (SEQ ID NO: 60) (upstream) and 5' TATGCTAGCTTATTTGGCCTTGAACCAACTG 3' (SEQ ID NO: 59) (downstream). The PCR fragment was digested with NheI and SpeI and inserted into the compatible NheI site of γ42.NheI-S to generate γ.γb::2A::βb (Figure 4E).

15

Inoculation protocol.
        See Example 1.

Results and discussion.
20   Another strategy to produce heterologous proteins that are not fused to virus proteins was to replace the βb ORF with that of the heterologous gene. Substitution of the GFPc3 gene for the βb gene (Figure 4D) resulted in low to moderate GFPc3 expression in protoplasts (inoculation number 8, Table 2). However, due to the lack of the βb gene, a required movement protein, these viruses were not infectious and

25   GFPc3 was not observed *in planta*. Therefore, to complement the lost movement function, βb was fused to the C-terminus of the γb protein, either with or without the 2A cleavage sequence (Figure 4E). Although γb::2A::βb (inoculation number 10, Table 2) was able to restore movement, it was only weakly infectious and did not confer systemic expression of GFP. It is possible that a βb::2A::γb fusion would be

30   more robust, but this has not been constructed or tested. If successful, this would provide another opportunity for systemic expression of two heterologous proteins simultaneously in barley.

Unlike the γ.γb::2A::βb virus, the virus expressing the γb::βb fusion from the γ

RNA did not complement the βb deletion (inoculation number 12, Table 2). This

suggests that the fusion of γb to the N-terminal of βb inactivates βb. The interference

of γb with βb when fused is supported by a comparison of the γb::2A::βb and γb::βb

5   fusions on the β RNA (inoculation number 13 and 14, Table 2), in which the 2A

sequence is also required for systemic infection. This effect appears to be specific to

the γb protein as GFPc3 did not interfere with βb activity when the GFPc3::βb fusion

was expressed from the β RNA (inoculation number 15, Table 2).

10   EXAMPLE 8

Expression of Heterologous proteins from Four-Component BSMV Based on RNA γ.

Construction of viral vectors.

         To generate the plasmid γ.Δγa.Δγb.GFPc3 (Figure 5A), γ.Δγb.GFPc3 was

15   digested with EcoRV and religated to remove the 1306 bp EcoRV fragment in the γa

ORF. To generate the plasmid γ.Δγa (Figure 5B), γ.42 was digested with EcoRV and

religated to remove the 1306 bp EcoRV fragment in the γa ORF.

Inoculation protocol.

20   See Example 1.

Results and discussion.

         To express heterologous proteins without fusion to viral proteins, two novel

four component strategies, with duplicated γ RNAs, were employed. Genetic

25   modifications to the fourth BSMV γ RNA allowed the expression of GFPc3 during

co-infections with BSMV α, ß, and γ RNAs.

         The four component satellite approach expresses high levels of GFPc3 in

protoplasts, but not in systemically infected plants. A virus whose fourth component

contained a γa deletion and a GFPc3 substitution of γb (Figure 5A) accumulated 2-5

30   fold more GFPc3 than the γb::GFP fusion virus in protoplasts (compare inoculation

number 3 with 6, Table 3), and amounts equivalent to TMV SBS5::GFP (compare

inoculation number 6 with 2, Table 3; and lane 3 with 1, Figure 5D). Although this virus was systemically infectious, it did not express GFPc3 systemically, probably due to the loss of the fourth component.

To increase the possibility that four components would be maintained during systemic expression, a complementary four component approach was also tested. In this approach the two γ RNAs had complementary viral gene deletions; one contained only the γb gene while the other contained the γa gene (Figure 5B). These two γ RNAs, when combined with the α and β RNAs, produced an attenuated systemic infection in barley (inoculation number 9, Table 3). To use this system to express a heterologous protein, GFPc3 was added to the γ.Δγb RNA in the γb slot (Figure 5C). This approach resulted in moderate levels of GFPc3 expression in protoplasts (inoculation number 12, Table 3), but was not systemically infectious.

Patent

Table 3*

#	Inoculum — Test Constructs 1	2	3	wt α	wt β	wt γ	GFP expression Tobacco Protoplast	GFP expression Barley Plants	Mosaic Plants
1	none			X	X	X			heavy
2	TMV-SBS5 GFPc3						*****		
3	γ.γb.GFPc3			X	X		****	systemic	heavy
4	γ.γb.GFPc3		β.Δβa	X			*****	bright systemic	heavy
5	γ.Δγa.Δγb.GFPc3			X	X				
6	γ.Δγa.Δγb.GFPc3			X	X	X	*****		hvy/mod
7	γ.Δγb			X	X				light
8		γ.Δγa		X	X				
9	γ.Δγb	γ.Δγa		X	X				light
10	γ.Δγb.GFPc3			X	X	X	***	.	heavy
11	γ.Δγb.GFPc3			X	X		**		
12	γ.Δγb.GFPc3	γ.Δγa		X	X		***		
13	γ.Δγa.Δγb.GFPc3		β.Δβa	X		X	******		heavy
14	γ.Δγb.GFPc3		β.Δβa	X		X	****		heavy
15	γ.Δγb.GFPc3		β.Δβa	X			***		
16	γ.Δγb.GFPc3	γ.Δγa	β.Δβa	X			*****		
17	γ.Δγa.Δγb.GFPc3	γ.Δγa.Δγb.GFPc3	β.Δβa	X		X	*******	.	heavy

*See Table 1 for explanation of abbreviation/symbols.

As mentioned above, replacement of the wild-type RNA β was with a βa deletion mutant appeared to increase expression from the γb subgenomic promoter. In protoplasts, the use of the βa deletion mutant was found to increase GFPc3 expression 2-5 fold for the four-component satellite approach (compare inoculation number 6 with 13, Table 3; and lane 3 with 4, Figure 5D), and 10-fold for the four component complementary approach (compare inoculation number 12 with 16, Table 3).

However, the βa deletion did not confer systemic infectivity to either the complementary fourth component system or systemic GFPc3 expression to the satellite system.

By doubling the dosage of the fourth GFPc3 bearing RNA relative to the other RNAs, an additional 2-5 fold increase in GFPc3 accumulation was observed (compare inoculation number 13 to 17, Table 3; and lane 4 with 5, Figure 5D). This result suggests that this four component system could be used to express high levels of multiple heterologous proteins in protoplasts from both mono and dicot species. This

could be tested by co-transfecting protoplasts with α, β.Δβa, γ and multiple satellite γ RNAs, each expressing a different heterologous protein (e.g. GFP, GUS, CAT, etc.) and transfecting protoplasts. If this were successful, this multi-gene vector system could be used to express various gene combinations to achieve the synthesis of novel

5   compounds and for gene discovery.

Many plant secondary metabolites accumulate to low levels in cell culture lines, compared with the native plant due to differential gene expression in various tissues. For example, many *N. tabacum* cell lines accumulate low (close to undetectable) levels of nicotine (data not shown). Several genes could be introduced

10   into virus expression vectors to add in enzymes or boost expression of functional enzymes that would work to produce complex metabolites. An example of this would be to boost scopolamine biosynthesis in *Nicotiana* (or other plant) protoplasts. Scopolamine is a secondary metabolite that is synthesized in the root pericycle of *Hyocyamus niger* plants and is a useful plant medicinal compound (Kanegae, *et al.*,

15   1994). However its accumulation is not significant in many other members of the Solanaceae. The essential genes that contribute to scopolamine synthesis can be cloned and added into separate fourth component satellite constructs, including: phenylalanine ammonia lyase (PAL; phenylalanine biosynthesis), putrescine N-methyl transferase and amine oxidase (putrescine metabolism), and tropinone

20   reductase, hyoscyamine 6S – hydroxylase epoxidase and hyoscyamine 6S – hydroxylase dioxygenase all particularly involved in scopolamine biosynthesis. Over expression of key enzymes, in the feeding pathways (phenylalanine and putrescine biosynthesis) or in the direct synthesis of scopolamine, using the four (or more) component BSMV expression system (described above) would be expected to divert

25   carbon flow from phenylalanine and putrescine synthesis and lead to an accumulation of scopolamine in the *Nicotiana* cells transfected with these replicating expression vectors. This method could be applied to many different systems where the expression of multiple genes would be required for functionality. It also could include the expression of particular processing enzymes and a substrate protein to produce an

30   active product. In addition, protease inhibitors could be expressed from one component while other components express protein products that are sensitive to protease activity (and thereby inhibited by the protease inhibitor).

EXAMPLE 9

The use of the BSMV vector to silence genes systemically in monocot species.

Construction of monocot viral vectors containing partial barley phytoene desaturase

5   cDNAs.

Partial cDNAs encoding phytoene desaturase (PDS) were amplified from

black hulless barley leaf tissue RNA by RT-PCR using oligonucleotides pairs 193 5'

ATATTAATTAACTAAACCCATATTGCTTGAGGCAA 3' (SEQ ID NO: 61)

(upstream) and 194 5' TATGCGGCCGCCTAGTGTAGTCACCAGCTAGATAG 3'

10   (SEQ ID NO: 62) (downstream), pairs 193 5'

ATATTAATTAACTAAACCCATATTGCTTGAGGCAA 3' (SEQ ID NO: 61)

(upstream) and 196 5' TATGCGGCCGCCTACTTTCAGGAGGATTACCATCC 3'

(SEQ ID NO: 63) (downstream), pairs 195 5'

ATATTAATTAACTGGATGAAAAAGCAGGGTGTTCC 3' (SEQ ID NO: 64)

15   (upstream) and 194 5' TATGCGGCCGCCTAGTGTAGTCACCAGCTAGATAG

3'(SEQ ID NO: 62) (downstream), and pairs 195 5'

ATATTAATTAACTGGATGAAAAAGCAGGGTGTTCC 3' (SEQ ID NO: 64)

(upstream) and 196 5' TATGCGGCCGCCTACTTTCAGGAGGATTACCATCC 3'

(SEQ ID NO: 63) (downstream). The PCR fragments containing portions of the barley

20   PDS cDNA, were digested with PacI and NotI, and inserted between the PacI and

NotI sites of γ.γb.st.P/N-zeo-B and γ. γb.st.N/P-zeoB (Figure 1C). The insertion of the

partial cDNA fragments eliminated the Zeocin resistance gene and created plasmids

with PDS inserts in the positive orientation (γ.γb.st.P/N-bPDS1, γ.γb.st.P/N-bPDS2,

γ.γb.st.P/N-bPDS3, and γ.γb.st.P/N-bPDS4) and negative orientation (γ.γb.st.N/P-

25   bPDS1-as, γ.γb.st.N/P-bPDS2-as, γ.γb.st.N/P-bPDS3-as, and γ.γb.st.N/P-bPDS4-as)

(Figure 5E). These BSMV::PDS hybrid RNAs are designed to express untranslated

PDS nucleotide sequences as fusions to the γ subgenomic RNA 1.

Construction of monocot viral vectors containing partial rice phytoene desaturase

30   cDNAs.

Partial cDNAs encoding phytoene desaturase (PDS) were amplified from rice

leaf tissue RNA by RT-PCR using oligonucleotides pairs 193 5'

ATATTAATTAACTAAACCCATATTGCTTGAGGCAA 3' (SEQ ID NO: 61)

(upstream) and 194 5' TATGCGGCCGCCTAGTGTAGTCACCAGCTAGATAG

3'(SEQ ID NO: 62) (downstream), pairs 193 5'

ATATTAATTAACTAAACCCATATTGCTTGAGGCAA 3' (SEQ ID NO: 61)

5   (upstream) and 196 5' TATGCGGCCGCCTACTTTCAGGAGGATTACCATCC

3'(SEQ ID NO: 63) (downstream), pairs 195 5'

ATATTAATTAACTGGATGAAAAAGCAGGGTGTTCC 3' (SEQ ID NO: 64)

(upstream) and 194 5' TATGCGGCCGCCTAGTGTAGTCACCAGCTAGATAG 3'

(SEQ ID NO: 62) (downstream), and pairs 195 5'

10   ATATTAATTAACTGGATGAAAAAGCAGGGTGTTCC 3' (SEQ ID NO: 64)

(upstream) and 196 5' TATGCGGCCGCCTACTTTCAGGAGGATTACCATCC 3'

(SEQ ID NO: 63) (downstream). The PCR fragments containing portions of the rice

PDS cDNA, were digested with PacI and NotI, and inserted between the PacI and

NotI sites of γ.γb.st.P/N-zeo-B and γ. γb.st.N/P-zeoB (Figure 1C). The insertion of the

15   partial cDNA fragments eliminated the Zeocin resistance gene and created plasmids

with PDS inserts in the positive orientation (γ.γb.st.P/N-rPDS1, γ.γb.st.P/N-rPDS2,

γ.γb.st.P/N-rPDS3, and γ.γb.st.P/N-rPDS4) and negative orientation (γ.γb.st.N/P-

rPDS1-as, γ.γb.st.N/P-rPDS2-as, γ.γb.st.N/P-rPDS3-as, and γ.γb.st.N/P-rPDS4-as)

(Figure 5E). These BSMV::PDS hybrid RNAs are designed to express untranslated

20   PDS nucleotide sequences as fusions to the γ subgenomic RNA 1.

Construction of monocot viral vectors containing full length and partial maize

phytoene desaturase cDNAs.

Partial cDNAs encoding phytoene desaturase (PDS) were amplified from corn

25   leaf tissue RNA by RT-PCR using oligonucleotides pairs 175 5'

ATATTAATTAACATGGACACTGGCTGCCTGTC 3' (SEQ ID NO: 65) (upstream)

and 180 5' TATGCGGCCGCCTACAAAGCAATCAAAATGCACTG 3'(SEQ ID

NO: 66) (downstream) encoding PDS Met^1-Leu^{290}, pairs 177 5'

ATATTAATTAACAAGGTAGCTGCTTGGAAGGATG 3' (SEQ ID NO: 67)

30   (upstream) and 178 5' TATGCGGCCGCCTAGCAGGTTACTGACATGTCTGC 3'

(SEQ ID NO: 68) (downstream) encoding PDS Lys^{134}-Cys^{431}, and pairs 179 5'

ATATTAATTAACCAGTGCATTTTGATTGCTTTG 3' (SEQ ID NO: 69)

(upstream) and 176 5' TATGCGGCCGCCTAAGATGGGACGGGAACTTCTCC 3'
(SEQ ID NO: 70) (downstream) encoding PDS Gln^{284}-Ser^{571}. The full length cDNA
encoding phytoene desaturase (PDS) was amplified from corn leaf tissue RNA by RT-
PCR using oligonucleotides pairs 175 5'

5   ATATTAATTAACATGGACACTGGCTGCCTGTC 3' (SEQ ID NO: 65) (upstream)
and 176 5' TATGCGGCCGCCTAAGATGGGACGGGAACTTCTCC 3' (SEQ ID
NO: 70) (downstream) encoding PDS Met^1-Ser^{571}. The 0.8 Kb PCR fragments
containing portions of the corn PDS cDNA, and the 1.6 Kb PCR fragment containing
the full length corn PDS ORF, were digested with PacI and NotI, and inserted

10   between the PacI and NotI sites of γ.γb.st.P/N-zeo-B and γ. γb.st.N/P-zeoB (Figure
1C). The insertion of the partial and full length PCR fragments eliminated the Zeocin
resistance gene and created plasmids with PDS inserts in the positive orientation
(γ.γb.st.P/N-mPDS-N, γ.γb.st.P/N-mPDS-M, γ.γb.st.P/N-mPDS-C, and γ.γb.st.P/N-
mPDS) and negative orientation (γ.γb.st.N/P-mPDS-N-as, γ.γb.st.N/P-mPDS-M-as,

15   γ.γb.st.N/P-mPDS-C-as, and γ.γb.st.N/P-mPDS-as) (Figure 5E). These BSMV::PDS
hybrid RNAs are designed to express untranslated PDS nucleotide sequences as
fusions to the γ subgenomic RNA 1.

Construction of monocot viral vectors containing full-length and partial *N.*

20   *benthamiana* phytoene desaturase cDNAs.

        The full length cDNA encoding *N. benthamiana* PDS was amplified from the
plasmid pPDS Nb2, which contains an insert with the sequence depicted by SEQ ID
NO: 3 of U.S. Patent No. 5,539,093. To amplify the nucleic acid encoding PDS Met^1-
Val^{580} (encompassed by SEQ ID NO: 3 of U.S. Patent No. 5,539,093) oligonucleotides

25   pairs 189 5' ATATTAATTAACATGCCCCAAATTGGACTTGTTTC 3' (SEQ ID
NO: 71) (upstream) and 190 5'
TATGCGGCCGCCTACTAAACTACGCTTGCTTCTGC 3' (SEQ ID NO: 72)
(downstream) were used.   cDNA was placed under the control of the BSMV γb
subgenomic promoter by subcloning into the PacI and NotI sites of both γ.γb.st.P/N-

30   zeo-B and γ. γb.st.N/P-zeoB (Figure 1C). The partial cDNAs encoding *N.*
benthamiana PDS were amplified from the plasmid pPDS Nb2 using oligonucleotides
pairs 189 5' ATATTAATTAACATGCCCCAAATTGGACTTGTTTC 3' (SEQ ID

NO: 71) (upstream) and 192 5'
TATGCGGCCGCCTAGGGTTTATGAAGTTAAGTGCC 3' (SEQ ID NO: 73)
(downstream) encoding PDS Lys^{134}-Cys^{431}, and pairs 191 5'
ATATTAATTAACAAGGCACTTAACTTCATAAACCC 3' (SEQ ID NO: 74)

5   (upstream) and 190 5' TATGCGGCCGCCTACTAAACTACGCTTGCTTCTGC3'
(SEQ ID NO: 72) (downstream) encoding PDS Gln^{284}-Ser^{571}. The 1.7 Kb PCR
fragment containing the full length *N. benthamiana* PDS and the 0.9 Kb PCR
fragments containing portions of the *N. benthamiana* PDS cDNA were digested with
PacI and NotI and inserted between the PacI and NotI sites of γ.γb.st.P/N-zeo-B and

10   γ. γb.st.N/P-zeoB (Figure 1C). The insertion of the partial and full-length cDNA
fragments eliminated the Zeocin resistance gene and created plasmids with PDS
inserts in the positive orientation (γ.γb.st.P/N-nPDS1, γ.γb.st.P/N-nPDS2, and
γ.γb.st.P/N-nPDS3) and negative orientation (γ.γb.st.N/P-nPDS1-as, γ.γb.st.N/P-
nPDS2-as, and γ.γb.st.N/P-nPDS3-as) (Figure 5E). These BSMV::PDS hybrid RNAs

15   are designed to express untranslated PDS nucleotide sequences as fusions to the γ
subgenomic RNA 1.

Inoculation protocol.

    Infectious BSMV RNAs from cDNA clones were prepared by *in vitro*

20   transcription using T7 DNA-dependent RNA polymerase (Ambion) as decribed
previously (Petty, *et al*; 1989). Transcripts of each of the three BSMV genomes were
mixed in a 1:1:1 ratio. A 7.5 μl aliquot of the transcription mix was combined with
45 μL of FES and directly applied to 12 day old black hulless barley plants. Barley
cultivars used include: Abrabische, Agio, Alexis, Atlas, B83, Berwick, Binder, Black

25   Hulless, Chariot, Cooper, Delta, Derkado, Golden Promise, Gotlan DS, Haise, Hanna,
Intensiv, Irish Archer, Kenia, Lyallpur, Monte Cristo, Opal, Optic, Prisma, Spring
Wheat, Tankard, Tern, Tyne, Vivet, and Vollkorn.

Purification and analysis of carotenoids from transfected barley plants.

30   The carotenoids were isolated from 50 mg of systemically infected leaf tissue
18 days post inoculation and analyzed by HPLC chromatography. Carotenoids were
extracted in the dark in methanol and identified by their peak retention time and

absorption spectra on a Zorbax 4.6 x 15 cm C-18 column using acetonitrile/methanol/2-propanol (85:10:5) as a developing solvent at a flow rate of 2 ml/min. They had identical retention times to a synthetic phytoene standard and β-carotene standards from tomato and carrot.

5

Preparation of cDNA library in monocot vector.

Oryza sativa ecotype Indica and Japonica (0) seeds were sown and the meristem, root, leaves and panicles were collected at appropriate time points. Tissue was washed in deionized water and frozen in liquid nitrogen. High quality total RNA

10 was isolated using a hot borate method (Wilkins and Smart, 1996). mRNA was isolated from total RNA using oligo (dT)$_{25}$ magnetic beads (Miltenyi) according to manufacturer's instructions. The purified polyA$^+$ RNA was then used for cDNA synthesis by conventional strand replacement reactions (Okayama and Berg, 1982; Gubler and Hoffman, 1983). Double strand cDNA was synthesized either with NotI-

15 (dT)$_{25}$ primer based on the manufacturer's instruction (Gibco-BRL superscript system). Typically, 5 μg of poly A$^+$ RNA was annealed and reverse transcribed at 37°C with Superscript II reverse transcriptase. Double stranded cDNAs were ligated to a 500 to 1000-fold molar excess SalI adaptor, restriction enzyme NotI digested and size-selected by column fractionation.

20 cDNAs from four different tissues: 7 weeks root, 7 weeks leaf, meristem and mixed panicles were pooled and then cloned directionally into the SalI-NotI sites of the BSMV expression vector, γ.γb.st.P/N-zeo-B and γ. γb.st.N/P-zeoB (Figure 1C). The ratio between the vector and insert is 280ng to 60 ng and the transformation efficiency is ~ 2 x 10^6 cfu/μg. Each ligation was transformed into chemically

25 competent *E. coli* cells, DH5 α according to manufacturer's instruction (Gibco-BRL). To ascertain the cloning efficiency of cDNA into each vector and the average insert size, 96 random colonies were picked and grown by standard molecular biology methods. DNA was isolated from bacteria using a Qiagen BioRobot 9600. DNA was digested with Not I and PacI restriction endonucleases (recognition sites flank the

30 cDNA insertion). The digestions were separated on agarose gels and visualized by ethidium bromide staining. Approximately 600 independent clones were analyzed by

restriction digestion as described above. The average insert size in the vector was ~1 kb, with many inserts with 2 kb or greater sized inserts.

Results and discussion.

5   BSMV was used to silence the endogenous phytoene desaturase (PDS) gene in barley. Black Hulless barley plants were inoculated with BSMV RNA α and β.Δβa, and a γ RNA designed to express one of several portions of the rice PDS cDNA (γ.γb.st.P/N.bPDS1, γ.γb.st.P/N.bPDS2, γ.γb.st.P/N.bPDS3, γ.γb.st.P/N.bPDS4, γ.γb.st.P/N.bPDS1-as, γ.γb.st.P/N.bPDS2-as, γ.γb.st.P/N.bPDS3-as, and

10   γ.γb.st.P/N.bPDS4-as: collectively referred to as BSMV::mPDS). The various BSMV::bPDS hybrid viruses spread throughout the non-inoculated leaves as determined by the appearance of mosaic symptoms and by local lesion assay on *Chenopodium amranticolor*. The presence of the PDS insert was confirmed by Reverse Transcriptase (RT)-PCR using primers specific to the BSMV γ RNA and

15   flanking the inserted PDS cDNA fragment. The initial viral symptomology (1-7 days post inoculation) resulting from the BSMV::bPDS hybrid viruses was indistinguishable from a wild type BSMV infection. However, between 8-10 days post inoculation, the BSMV::bPDS plants began to exhibit streaks and patches of unusually white tissue. The affected areas lacked the necrosis or desiccation that is

20   often associated with BSMV induced bleaching and, instead, appeared more like the bleached tissue found in plants treated with the chemical inhibitor of PDS, norflurazon. These white streaks were observed to some degree in all the BSMV::bPDS infected barley plants, regardless of whether the PDS fragment was sense or antisense. The PDS gene is part of the carotenoid biosynthetic pathway,

25   which produces colored compounds that protect the green pigment, chlorophyll from photobleaching (Bartley and Scolnik, 1995). The presence of the bleached tissues suggested that the endogenous PDS gene was no longer functional in these tissues.

      In Black Hulless, phytoene desaturase gene (PDS) silencing is first observed as streaks of bleached tissue in the uppermost leaf (leaf 3 or 4, numbered from the

30   base) between 8-11 days post inoculation (dpi). The accumulation of phytoene, the substrate for PDS, can also be detected in these leaves at this time by HPLC analysis. Phytoene accumulation can be measured as the number of Area Units (AU) under the

HPLC retention peak. Uninoculated barley plants, and barley plants infected with wild-type BSMV or BSMV::GFP (Figure 2A) have an average of approximately 60 AU of phytone, and can occasionally be as high as 200 AU (inoculation 9-11, Table 4, Figures 6-7). In plants inoculated with BSMV::bPDS hybrid viruses, average

5 phytoene levels accumulate to 5-40 fold above the control plant, depending on time post infection, leaf position, and the PDS fragment (inoculations 1-8, Table 4, Figures 8-10). These changes in phytoene levels were similar to that observed with norflurazon treated plants (Table 4, Figures 11-13), thereby providing biochemical evidence for the genetic silencing of the PDS endogene. Bleached areas continue to

10 appear and expand in leaves 4, 5 and sometimes 6, over the period from 12 to 20 dpi, and occasionally up to 25 dpi. After 18-20 dpi, phytoene levels begin to become lower (Table 4). Phytoene accumulation and photobleaching are spatially and temporally related, and unevenly distributed throughout BSMV-bPDS infected plants. Leaves with visual bleaching show high levels of phytoene accumulation, while other leaves

15 on the same plant may or may not have any phytoene accumulation.

Our results demonstrating bleaching phytoene accumulation in barley plants transfected with partial antisense and sense phytoene desaturase suggest that plant viral vectors can be used to manipulate biosynthetic pathways in monocots through cytoplasmic inhibition of endogenous gene expression.

20

Table 4

Inoculum				Phytoene accumulation in HPLC area units			
viral RNAs				(average of 2 plants, uppermost leaf)			
#	Test Constructs	α	β.Δβa	11dpi	14dpi	17dpi	21dpi
1	γ.γb.st.bPDS1-A6	X	X	995	2425	804	1495
2	γ.γb.st.bPDS1-as-B10	X	X	1429	1280	1739	104
3	γ.γb.st.bPDS2-G11	X	X	2020	1434	379	986
4	γ.γb.st.bPDS2-as-C5	X	X	1676	775	nt	658
5	γ.γb.st.bPDS3-D5	X	X	371	1382	1419	267
6	γ.γb.st.bPDS3-as-E8	X	X	465	1247	1208	145
7	γ.γb.st.bPDS4-C1	X	X	1653	1161	1375	1342
8	γ.γb.st.bPDS4-as-A4	X	X	1265	1449	506	316
9	γ.γb.GFPc3	X	X	53	47	67	68
10	γ	X	X	135	10	80	41
11	Mock			59	71	30	10
12	Norflurazon			2355	2180	2439	2205

Endogene homology and BSMV-induced gene silencing.

Experiments were designed to evaluate the effect of gene homology of the insert in the virus vector with the endogene that is targeted for silencing. Various PDS gene fragments from barley (*Hordeum vulgare*), rice (*Oryza sativa*), corn (*Zea mays*), and *N. benthamiana* were cloned into γ.γb.st.P/N-zeo-B and γ. γb.st.N/P-zeoB

5 (Figure 1C). This produced in an array of viruses expressing sense and antisense PDS fragments, each of which was inoculated onto black hulless barley plants. All of the viruses bearing rice and corn PDS fragments were able to silence barley PDS, as judged by the appearance of bleached tissue and the accumulation of phytoene (Table 5). Thus BSMV could heterologous PDS fragments to silence the endogenous barley

10 PDS. For barley, rice and corn fragments, increased homology between virus encoded PDS fragments and genomic encoded PDS results in increased bleaching intensity, phytoene accumulation, and consistency from plant to plant (Table 5). Viruses expressing *N. benthamiana* PDS fragments, those least homologous to barley did not silence barley PDS. Together these results suggest that homology with the

15 endogenous gene may be a limiting factor in BSMV mediated gene silencing in barley.

Table 5

BSMV::PDS			Phytoene Area Units	
Insert*	Insert source	Homology**	average***	standard error
bPDS	*Hordeum vulgare*	100%	1231	100
rPDS	*Oryza sativa*	90%	897	150
mPDS	*Zea mays*	88%	497	136
nPDS	*Nicotiana benthamiana*	74%	90	8
GFP	na	na	105	20
none	na	na	62	3

na: not applicable
* Four insert fragments tested, each in both orientation, for each PDS gene **(Table)**
** nucleatide homolgy to barley PDS sequence (partial)
*** Average area units based on 32 samples from 3 timepoints for each insert

The ability to silence endogenous barley genes using cDNA fragments from a

20 heterologous species suggests that BSMV could be used to discover new genes based on functional knockout of the barley homolog. To demonstrate the utility of this vector as a gene discovery tool, a library of BSMV γ RNAs expressing rice cDNAs, in the sense and antisense orientation, was generated in γ.γb.st.P/N-zeo-B and γ. γb.st.N/P-zeoB (Figure 1C). The γ::cDNA library was generated in a 96 well

25 format to aid in handling and inoculated onto trays of 96 plants. By screening

approximately 1100 unique cDNA inserts expressed by BSMV::cDNA in barley, 25 novel visible phenotypes were detected. To date three of these phenotypes have been confirmed.

5   To this point, experiments have been conducted using a β RNA with a coat protein deletion (β.Δβa). To determine the effect of the BSMV coat protein on BSMV mediated silencing of PDS, experiments were performed with the wild type β RNA as well. Experiments with BSMV expressing corn PDS suggest that BSMV mediated gene silencing is enhanced by the deletion of the βa gene (coat protein) (Table 6). In general, coat protein deletion increases both the frequency and extent of

10   both bleaching and phytoene accumulation (Table 6) in black hulless barley.

Table 6

Inoculum		Fraction of plants showing PDS silencing phenotype at 19 dpi		
#	Test Constructs	β	β.Δβa	none
1	γ.γb.st.PDS-n-B5	0/6	2/6	
2	γ.γb.st.PDS-n-B7	1/6	2/6	
3	γ.γb.st.PDS-m-C5	5/6	6/6	
4	γ.γb.st.PDS-m-C9	2/6	5/6	
5	γ.γb.st.PDS-c-D6	1/6	1/6	
6	γ.γb.st.PDS-c-D12	1/6	3/6	
7	γ.γb.st.PDS-nR-E1	0/6	0/6	
8	γ.γb.st.PDS-nR-E2	1/6	0/6	
9	γ.γb.st.PDS-mR-F2	3/6	3/6	
10	γ.γb.st.PDS-mR-F5	2/6	3/6	
11	γ.γb.st.PDS-cR-G8	1/6	3/6	
12	γ.γb.st.PDS-cR-G12	0/6	1/6	
13	γ.γb.P/N::c3-A5	0/6	0/6	
14	γ.γb.P/N::c3as-A2	0/6	0/6	
15	γ STP	0/6	0/6	
16	γ.γb.c3.ΔM-1	0/6	0/6	
17	mock			0/6
18	uninoculated, NF treated			2/2
total number of PDS silenced plants:		17	29	

BSMV vector expression and gene silencing in alternative Barley cultivars.

Data presented to this point has been using the BSMV vector in the Black Hulless cultivar of barley. However, the BSMV vector is capable of expression and endogene silencing in other cultivars of barley. Barley cultivars were screened for BSMV susceptibility, GFPc3 expression and PDS silencing by inoculating 2 plants each with wild-type BSMV, BSMV::GFPc3, and BSMV::bPDS4, both with and without the coat protein. BSMV infection and BSMV-mediated PDS silencing and GFPc3 fluorescence were observed in all cultivars tested. BSMV-mediated PDS silencing and GFPc3 expression are enhanced in all barley cultivars by the deletion of the βa gene (coat protein) from the vector (Table 7). In the absence of the coat protein, on average, a higher percentage of tissue has GFPc3 fluorescence and this tissue is brighter. In general, the deletion of the coat protein gene also increases virus pathology and movement. The increase in both GFPc3 accumulation and pathology are most pronounced in less susceptible barley cultivars. Initial testing with wild-type BSMV, γb::GFP and γb::bPDS (barley PDS gene) revealed several cultivars that appear to be improved hosts compared to black hulless (Table 7). Promising cultivars maintain high or higher levels of GFPc3 expression and PDS silencing, but have a reduction in virus pathology; these will require further testing on larger scale for confirmation.

Table 7

| Cultivar \ Inoculum | α, γ.γb.st.bPDS4 | | α, γ.γb.GFP | | |
	with β	with βΔβa	with β	with βΔβa	none
Gotlan DS	993	903			
Arabische	140	1455			
Vollkorn	167	684			
B83	1145	1250			
Derkado	104	705			
Tern	264	140			
Haisa	254	865			
Kenia	469	1412			
Delta	157	156			
Asio	2154	672			
Tankard	166	262			
Binder	361	1761			
Hanna	476	827			
Berwick	255	1428			
Intensiv	419	784			
Alexis	167	788			
Golden Promise	531	315			
Spring Wheat	256	721			
Lyallpur	355	789			
Irish Archer	487	1605			
Livet	331	1222			
Opal	574	1446			
Monte Cristo	1223	1238			
Cooper	999	1035			
Prisma	235	1026			
Optic	150	1199			
Atlas	450	1204			
Chariot	117	1243			
Tyne	421	992			
Black Hulless	810	665			
Black Hulless GFP			104	38	
Black Hulless mock					65
Black Hulless Uninoculated					40
Norflurazon					1576

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention.

All publications, patents, patent applications, and web sites are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent, patent application, or web site was specifically and individually indicated to be incorporated by reference in its entirety.

LITERATURE CITED

Ahlquist, P., Luckow, V., and Kaesberg, P. (1981). Complete nucleotide sequence of brome mosaic virus RNA3. J. Mol. Biol. 153:23-28.

5

Bartley, G.E., and Scolnik, P.A. (1995). Plant carotenoids: pigments for photoprotection, visual attraction, and human health. Plant Cell 7(7):1027-38.

Chaplin, P.J., Camon, E.B., Villarreal-Ramos, B., Fling, M., Ryand, M.D., and

10   Collins, R.A. (1999). Production of interleukin-12 as a self-processing 2A polypeptide. Interferon Cytokine Res. 19(3):235-41.

Choi I., Stenger D.C., Morris T.J., French R. (2000). A plant virus vector for systemic expression of foreign genes in cereals. The Plant Journal 23(4), 547-555.

15

Crameri A., Whitehorn E.A., Tate E, and Stemmer W.P. (1996). Improved green fluorescent protein by molecular evolution using DNA shuffling. Nature Biotechnol. 14 (3): 315-9.

20   DeFelipe, P., and Izquierdo, M. (2000). Tricistronic and tetracistronic retroviral vectors for gene transfer. Hum. Gene Ther. 11(13): 1921-31.

DeFelipe, P., Martin, V., Cortes, M.L., Ryan, M., and Izquierdo, M. (1999). Use of the 2A sequence from foot-and-mouth disease virus in the generation of retroviral

25   vectors for gene therapy. Gene ther. 6(2):198-208.

DeNoto, F.M., Moore,D.D., and Goodman, H.M. (1981) Nucleic Acids Res. 9, 3719-3730.

30   Digby, M.R. and Lowenthal, J.W. (1995) J. Interferon Cytokine Res. 15 (11), 939-945.

Donald, R.G., and Jackson A.O. (1998). The barley stripe mosaic virus gamma b gene encodes a multifunctional cycteine-rich protein that affects pathogenesis. Plant Cell 6(11):1593-606.

5   Donnelly, M.L., Gani, D., Flint, M., Monaghan, S., and Ryan, M.D. (1997) The cleavage activities of aphthovirus and cardiovirus 2A proteins. J. Gen Virol. 78 (Pt 1)13-21.

Gopinath, K., Wellink, J., Porta, C., Taylor, K.M., Lomonossoff, G.P., and van 10   Kammen A. (2000). Engineering cowpea mosaic virus RNA-2 into a vector to express heterologous proteins in plants. Virol. 267(2):159-73.

Graham J.S., Pearce G., Merryweather J., Titani K., Ericsson L. and, Ryan C.A. (1985). Wound-induced proteinase inhibitors from tomato leaves. I. The cDNA-15   deduced primary structure of pre-inhibitor I and its post-translational processing. J Biol. Chem. 260(11):6555-60.

Halpin, C., Cooke, S.E., Barakate, A., El Amrani, A., and Ryan, M.D. (1999) Self-processing 2A-polyproteins—a system for co-ordinate expression of multiple proteins 20   in transgenic plants. Plant J. 17(4):453-9.

Haupt, S., Duncan, G.M., Jackson, A.O., Holzberg, S., and Oparka, K.J. (2001) Sieve-element unloading in sink leaves of barley is symplastic. Plant Physiology (in press).
25

Jackson, A.O., and Hunter, B.G. (1989). Hordeivirus relationships and genome organization. *Annu. Rev. Phytopathol.* 27:95-121.

Jackson, A.O., Petty, I.T.D., Jones, R.W., Edwards, M.C., and French R. (1991). 30   Analysis of barley stripe mosaic virus pathogenicity. Sem. Virol. 2:107-19.

Jarvik J.W., and Telmer C.A. (1998) Epitope tagging. Annu Rev Genet. 1998;32:601-18.

Joshi, R.L., Joshi V., and Ow D.W. (1998). BSMV genome mediated expression of a foreign gene in dicot and monocot plant cells. EMBO. 9:2663-9.

5   Kanegae,T., Kajiya,H., Amano,Y., Hashimoto,T. and Yamada,Y. (1994) Plant Physiol. 105(2): 483-490.

Kokuho, T., Watanabe, S., Yokomizo, Y., and Inumaru, S. (1999) Production of biologically active, heterodimeric porcine interleukin-12 using a monocistronic

10   baculoviral expression system. Vet. Immunol Immunopathol. 72(3-4):289-302.

Li, F., Browning, G.F., Studdert, M.J., and Crabb, B.S. (1996). Equine rhinovirus 1 is more closely related to foot-and-mouth disease virus than to other picornaviruses. Proc. Natl. Acad. Sci. USA 93(3):990-5

15

Mattion, N.M., Harnish, E.C., Crowley, J.C., and Reilly, P.A. (1996) Foot-and-mouth disease virus 2A protease mediates cleavage in attenuated Sabin 3 poliovirus vectors engineered for delivery of foreign antigens. J. Virol. 70(11):8124-7.

20   McKinney HH, and Greeley, L.W., (1965). Biological characteristics of barley stripe mosaic virus strains and their evolution. Technical Bulletin U.S. Department of Agriculture 1324.

Palmenberg, A.C. (1990). Proteolytic processing of picornaviral polyprotein. *Annu.*
25   *Rev. Microbiol.* 44:603-23.

Palomar, M.K., Brakke M.K., and Jackson, A.O. (1977). Base sequence homology in the RNAs of barley stripe mosaic virus. Virol.77(2):471-80.

30   Petty, I.T., Hunter, B.G., Wei, N., and Jackson A.O. (1989). Infectious barley stripe mosaic virus RNA transcribed in vitro from full-length genomic cDNA clones. Virol.171(2):342-9.

Petty, I.T., French, R., Jones, R.W., and Jackson A.O. (1990). Identification of barley stripe mosaic virus genes involved in viral RNA replication and movement. EMBO Journal 9: 3453-3457

5   Pogue, G.P., Lindbo, J.A., Dawson, W.O., and Turpen, T.H. (1998) Tobamovirus transient expression vectors: tools for plant biology and high-level expression of foreign proteins in plants. In "Plant Molecular Biology Manual" (S.B. Gelvin and R.A. Schilperoot, eds) L4, pp. 1-27. Kluwer Academic Publishers, Dordrecht, The Netherlands.

10

Ryan, M.D., and Drew, J. (1994) Foot-and-mouth disease virus 2A oligopeptide mediated cleavage of an artificial polyprotein. EMBO Journal: 13(4):928-33,

Ryan, M.D., King, A.M., and Thomas, G.P. (1991). Cleavage of foot-and-mouth

15 disease virus polyprotein is mediated by residues located within a 19 amino acid sequence. 72(Pt 11):2727-32.

Weiland, J.J., and Edwards MC (1996). A single nucleotide substitution in the alpha a gene confers oat pathogenicity to barley stripe mosaic virus strain ND18. MPMI

20 9(1):62-67.

Zhou H., and Jackson, A.O., (1996). Analysis of *cis*-acting elements required for replication of barley stripe mosaic virus RNAs. Virol. 16(2):367-79.

25 Zhou, H., and Jackson, A.O., (1998). Expression of the barley stripe mosaic virus RNA beta "triple gene block. Virol. 16(2):367-79.